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For every woman presenting classic menopausal **hot flushes,** there is another who exhibits symptoms which are equally distressing but less clearly defined. For example, insomnia, easy fatigability, **headaches** may also be symptoms of declining ovarian function, but frequently are not so recognized because they occur long before and even years after menstruation ceases. When such is the case, the patient may be expected to **respond** to estrogen therapy. **"Premarin"** (complete equine estrogen-complex) produces not only prompt symptomatic relief but also imparts a gratifying **"sense of well-being."** It has no odor . . . imparts no odor. "Premarin"® estrogenic substances (water-soluble), also known as conjugated estrogens (equine), is supplied in tablet and liquid form.

ENDOCRINOLOGY

VOLUME 56 JANUARY, 1955 NUMBER 1

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CONCENTRATION OF PROGESTIN IN THE SERUM OF THE RABBIT DURING PREGNANCY, THE PUERPERIUM AND FOLLOWING CASTRATION[1,2]

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Department of Biological Sciences, Purdue University, Lafayette, Indiana

A COMPLETE understanding of the role of progesterone in pregnancy has been handicapped in the past by the lack of sensitive assay that could be used on small amounts of blood. Pregnanediol determinations are of limited significance since they measure the degradation products of progesterone and steroids of a similar nature and also reflect not only the level of the hormone in the blood but also variations in the metabolism of the hormone. Early attempts to measure the progestin level of the blood in women (Clauberg, Thiel and Ziecker, 1933; Block, 1936) and pregnant rabbits (Block, 1936) failed to reveal any activity. Block reported the presence of less than 1 rabbit unit in 12 liters of blood obtained from pregnant sows. The use of the rabbit-intrauterine test revealed the presence of progestin in the blood of human beings during pregnancy (Haskins, 1941; Hoffmann and von Lam, 1948) however the results were extremely variable.

Recently, a new bioassay for progesterone was described by Hooker and Forbes (1947) which is extremely sensitive, specific for progesterone (Hooker and Forbes, 1949) and is not affected by estrogens at the concentrations found in the blood during pregnancy (Zarrow and Neher, 1953).

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[1] Presented at the Federation Meetings, Chicago, April 1953.

[2] Aided by a grant from the Purdue Research Foundation.

[3] Presented in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department Veterinary Science, Purdue University, Lafayette, Indiana.

Recents reports have indicated a marked contradiction between the values obtained by the Hooker-Forbes assay and by chemical techniques (Haskins, 1950; Butt, Morris and Williams, 1951; Edgar, 1952, 1953). These results tend to show that the hormone measured by the Hooker-Forbes bioassay may not be progesterone. Consequently the term progestin will be used throughout this study, although in keeping with previous work, the progestational activity has been standardized against progesterone and will be expressed as μg. equivalents of progesterone.

Progestin levels have been determined during pregnancy in women (Forbes, 1951; Schultz, 1953), monkeys (Forbes, 1951), sheep (Neher and Zarrow, 1950) and mice (Forbes, 1948). The present study is concerned with progestin levels in the blood of rabbits during pregnancy and attempts to evaluate the role of ovary and the placenta in the rabbit. This species differs from women and sheep in that castration in the rabbit always leads to abortion or resorption whereas such is not always true in the latter two species. Observations were also made on the levels of progestin at parturition, during the puerperium and following castration and hysterectomy.

MATERIALS AND METHODS

Sexually mature, female rabbits of the grey chinchilla strain were used in these experiments. The animals were given food and water *ad libitum* in addition to lettuce thrice weekly. All operations were performed under Nembutal anaesthesia supplemented with ether and the ventral approach was used. Blood samples were obtained from the marginal ear vein, permitted to clot and the serum removed and stored at −20° C. until time of assay.

All assays for progestin were carried out on Rockland Swiss mice according to the Hooker-Forbes procedure (1947). Sexually mature mice were castrated and 16 days later the serum was injected into a 5 mm. segment of the uterus. Quantitative data were obtained by a serial dilution of the sera. The reaction was considered positive when the stromal nuclei of the uterine segment assumed an ovoid shape, displayed a conspicuous nucleolus and contained fine, evenly dispersed chromatin particles. Since very little change has been reported with regard to the bound progestin, only assays for free progestin were carried out.

RESULTS

Normal Pregnancy

Serum progestin levels were determined at various stages throughout pregnancy in 5 rabbits (Fig. 1). The levels were at a minimum at the time of mating and varied from 0.3 to 1.0 μg. progestin per ml. serum. A sharp rise was apparent on the 4th day which continued until the 12th day and then appeared to plateau at a concentration of 6 to 8 μg. per ml. until the 24th day. Thereafter the level increased slightly to 8 to 10 μg. at parturition. Determinations at 30 minutes postpartum failed to reveal any significant drop. The first significant change in progestin occurred at 12 hours

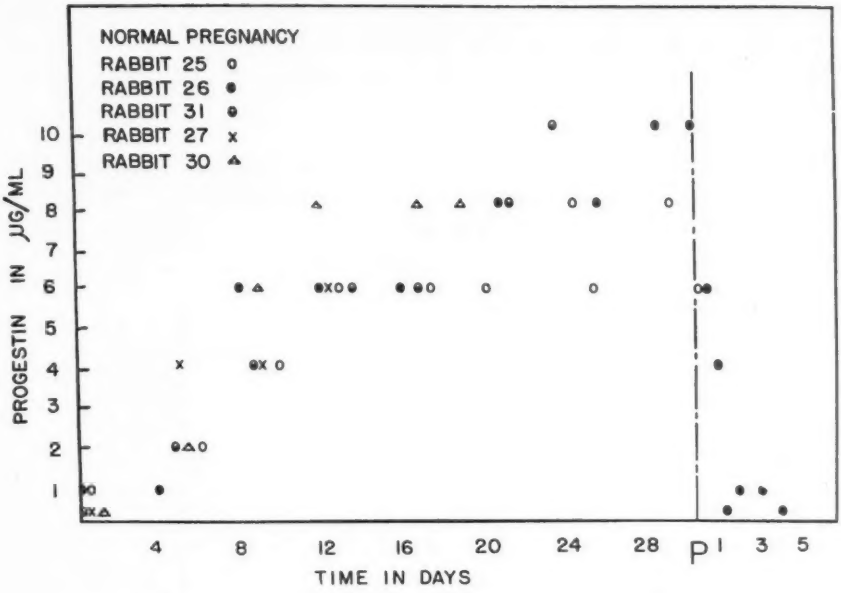

FIG. 1. Progestin levels in the blood of the rabbit during pregnancy.

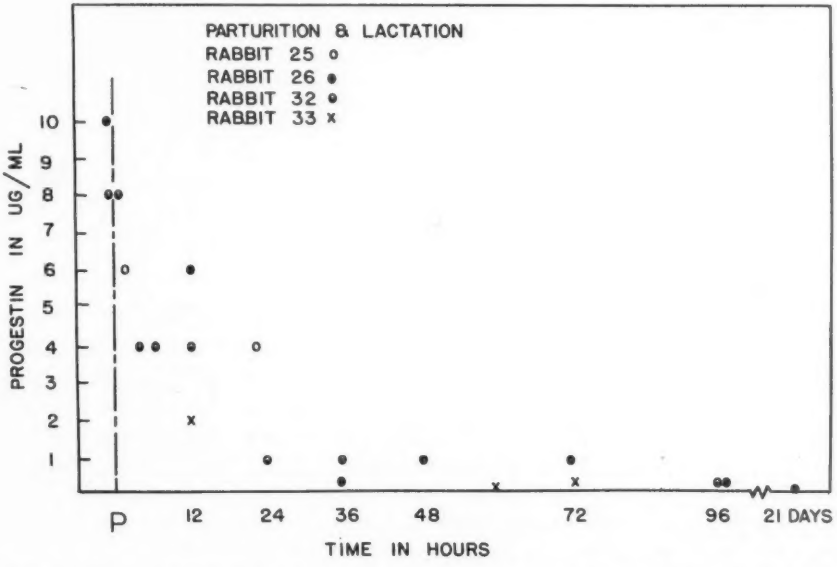

FIG. 2. Progestin levels in the blood of the rabbit at parturition and during lactation.

postpartum and involved a 40 to 50% decrease (Fig. 2). By 24 hours after delivery the porgestin level had returned to the pre-pregnancy level and these values (1.0 to 0.3 μg. per ml.) were maintained throughout the first 21 days of the lactation period.

Interrupted Pregnancy

Three rabbits were mated and the ovaries removed on the 12th, 19th, and 24th day of pregnancy. Progestin levels in the blood were determined

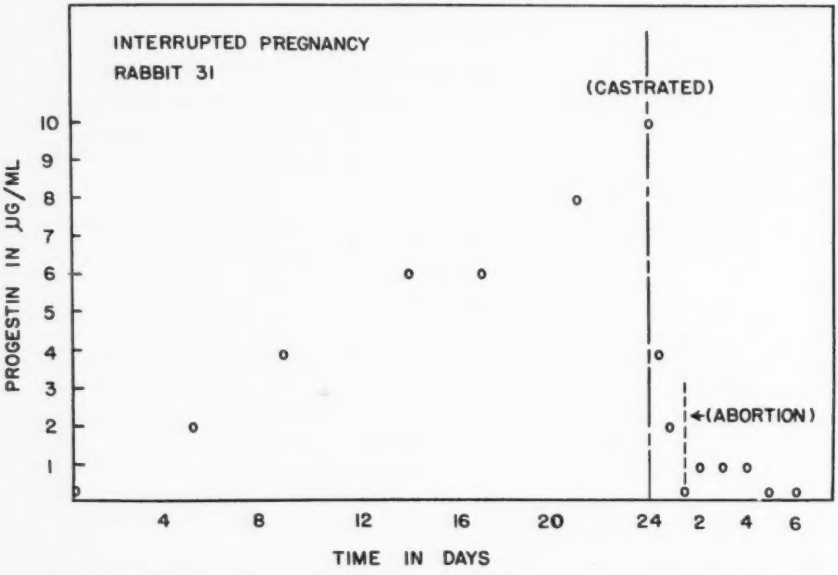

FIG. 3. The effect of castration on progestin levels in the pregnant rabbit. Rabbit No. 31 was castrated on the 24th day of pregnancy, at which time 10 μg. of progestin per ml. were present in the blood. The concentration of progestin decreased to 2 μg. per ml. in 24 hours and the animal aborted in 36 hours.

throughout this period and for 6 days following the removal of the ovaries. A 40 to 60% drop in the circulating progestin was noted 12 hours after the removal of the ovaries and an average decrease of 75% was observed in 24 hours. At this time a concentration of 1 to 4 μg. per ml. was found in the blood. All these animals aborted within 1 to 3 days after ovariectomy. A typical example of the effect of castration during pregnancy may be seen in Figure 3. Rabbit No. 31 showed a progressive increase in serum progestin throughout pregnancy reaching a level of 10 μg. per ml. on the 24th day. At this time the ovaries were removed and a drop in the progestin level was seen in 12 hours. The animal aborted 36 hours after castration at

which time the progestin level was down to 0.3 μg. per ml. Thereafter the concentration varied from 0.3 to 1.0 μg. per ml. throughout a 6 day post-castration period.

Castration and Hysterectomy

Two rabbits were castrated and hysterectomized on the 25th and 26th day of pregnancy respectively. The serum progestin varied between 8 and 10 μg. per ml. at the time of the operation and remained essentially unchanged for 6 hours (Fig. 4). However, a 50% drop was observed by the 8th hour after the operation with a further drop to 2 μg. per ml. by the 12th

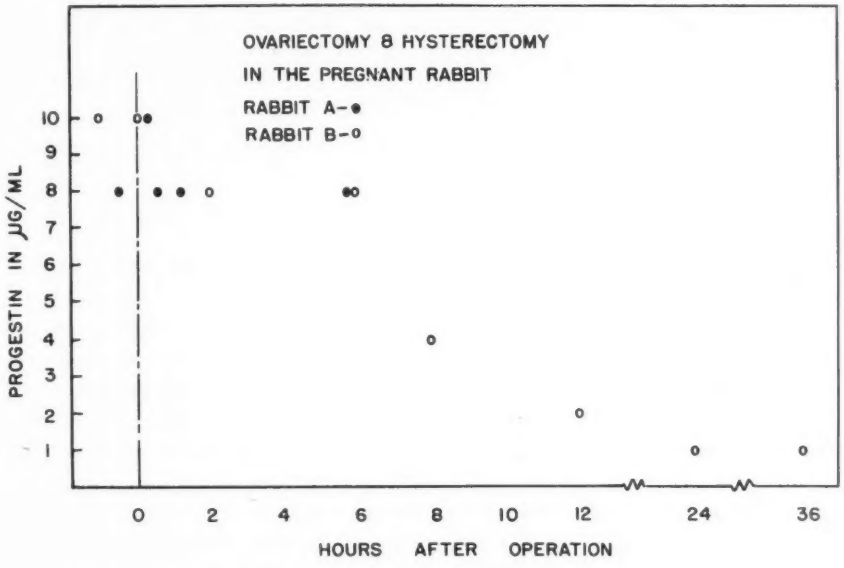

FIG. 4. The rate of fall in the serum progestin concentration in the pregnant rabbit following castration and hysterectomy.

hour. By the 24th hour, the progestin level had returned to the pre-pregnancy levels.

Disappearance of Progesterone from the Blood

Since the rate of disappearance of progestin from the blood after castration or castration and hysterectomy is identical with that seen at parturition, a comparison was made with the rate of disappearance following intravenous injection of progesterone. The hormone was administered intravenously in an amount that would result in a concentration of 10 μg. per ml. of serum. The plasma volume was determined by the Evans-blue

technique (Gregersen, 1944), and a 10% ethanol suspension of progesterone was used. A 40% drop in the serum progesterone level was observed within 4 minutes and an 80% drop in 8 minutes.

DISCUSSION

The progressive increase in the serum progestin levels of the rabbit during pregnancy is in agreement with the results obtained in the ewe (Neher and Zarrow, 1950) and the conclusions derived from pregnanediol studies in women (Browne, Henry and Venning, 1939). However, Forbes (1951) has indicated that no progressive rise in serum progestin, as determined by the Hooker-Forbes assay, is noted throughout pregnancy in women and monkeys. While more species need to be studied, it would appear that a species difference exists in that the primate fails to show a progressive increase in serum progestin during gestation. Furthermore, a biphasic curve has been observed in mice (Forbes, 1948) and a plateau in sheep (Neher and Zarrow 1950). The present data indicate a rapid rise in serum progestin levels of the rabbit until day 12 and a slower rise from day 12 till term. It is interesting to note that the period at which pregnancy in the ewe becomes independent of the ovary occurs during the time the progestin concentration levels off (Neher and Zarrow, 1950), whereas castration in the rabbit always leads to abortion or resorption. Since maintenance of pregnancy is independent of the ovary in primates (Hartman, 1941; Hartman and Corner, 1947; Melinkoff, 1950), this might explain the low progestin levels during gestation in the woman (Forbes, 1951). Both the rabbit and the ewe show high progestin levels although maintenance of pregnancy is independent of the ovary only during the last half of gestation in the ewe and dependent on the ovary throughout the entire gestation period in the rabbit.

No decrease in progestin levels was observed prior to parturition in the rabbit. As late as 30 minutes after delivery of the young, the progestin levels remained at the pre-delivery concentration. This is in agreement with observations on sheep (Neher and Zarrow, 1950) and the recent report of Bradshaw and Jessop (1953) on pregnanediol excretion in women. It would appear that contrary to the older concept, parturition occurs prior to any change in the concentration of progestin in the blood. This poses anew the problem of the mechanism of parturition and the manner whereby the dampening action of progesterone on uterine contractions and the oxytocic action of the posterior pituitary are controlled. It is clear that further work is needed before these problems can be answered.

The continued presence of minimal amounts of progestin in the blood during the puerperium is in agreement with observations on the monkey and women (Forbes, 1953). Observations in the rabbit indicate a variation in the concentration from 0 to 1 μg. per ml. from the 2nd to the 21st day

of lactation. Since concentrations of this order have been found in the male and identified as coming from the adrenal gland (Lazo-Wasem, Neher, Shoger and Zarrow, 1954) it is entirely possible that this may be the source of progestin during the puerperium.

The inability of the castrated, pregnant rabbit to maintain pregnancy has been known for some time (Corner, 1928). The present report confirms these observations and indicates that the first change following ovariectomy in the pregnant rabbit is a drop in the serum progestin levels followed by abortion. This would indicate that the ovary is the main source of progestin in this species and that abortion occurred as a result of insufficient hormone. Maintenance of pregnancy with progesterone in the castrated rabbit (Allen and Heckel, 1939) would tend to confirm the above. However, failure to observe a drop in serum progestin prior to parturition indicates that the significance of progestin must be further studied. It is entirely possible that a relative and not absolute insufficiency of the hormone may occur at parturition, i.e., the mass of fetus and placenta may increase at a much more rapid rate than the changes in hormone concentration. This possibility must await further study.

It has been previously shown that 90% of the activity is lost from the blood in 10 minutes after an intravenous injection of progesterone (Zarrow, Shoger and Lazo-Wasen, 1954). The present results indicate approximately a 50% drop in 4 hours after parturition with 24 to 36 hours required for a 90% decrease. Following castration in the pregnant rabbit, a 30 to 60% drop was observed in 12 hours. Since both these rates of decrease are much slower than that observed following intravenous injection of progesterone and since the placenta might have contributed to a slower rate in the castrated pregnant rabbit, several animals were castrated and hysterectomized on the 25th and 26th day of pregnancy. A 20% drop was observed in 6 hours and 90% drop in 24 hours. Following intravenous injection of progesterone in the castrated-hysterectomized rabbit, a 40% drop was observed in 2 minutes and an 80% drop in 6 minutes. It is apparent that the rate of loss of progesterone from the blood is markedly greater than that observed for progestin. This might be considered as further proof that the progestational hormone is not progesterone *per se*. It is likely that the progestational hormone in the blood may turn out to be a steroid very similar to progesterone or progesterone combined with another substance.

SUMMARY

Progestin levels were determined in the serum of rabbits by the Hooker-Forbes technique. A rapid increase was seen during the first 12 days of pregnancy and slower rise from the 12th day till term. A maximum concentration of 10 μg. per ml. of serum was noted just prior to parturition.

The serum progestin decreased after delivery of the young, indicating that a fall in the absolute progestin level is not essential for parturition. Castration led to a drop in serum progestin and abortion in all instances. It was noted that the rate of loss of progestin from the blood following a) parturition, b) castration of pregnant rabbits and c) castration and hysterectomy of pregnant rabbits was markedly slower than that observed after the intravenous injection of progesterone.

REFERENCES

ALLEN, W. M. AND G. P. HECKEL: *Am. Jour. Physiol.* **125**: 31. 1939.

BLOCK, P. W. *Endocrinology* **20**: 207. 1936.

BRADSHAW, T. E. T. AND W. J. E. JESSOP: *J. Endocrinol.* **9**: 427. 1953.

BROWNE, J. S. L., J. S. HENRY AND E. H. VENNING: *Am. J. Obst. & Gynecol.* **38**: 927. 1939.

BUTT, W. R., P. MORRIS, C. J. O. R. MORRIS AND D. C. WILLIAMS: *Biochem.* **49**: 434. 1951.

CLAUBERG, C., H. W. THEIL AND R. ZIECKER: *Arch. f. Gynak.* **152**: 61. 1933.

CORNER, G. W. *Amer. J. Physiol.* **86**: 74. 1928.

EDGAR, D. G. *Nature* **170**: 543. 1952.

EDGAR D. G. *J. Endocrinol.* **10**: 54. 1953.

FORBES, T. R. *Anat. Rec.* **100**: 661. 1948.

FORBES, T. R. *Endocrinology* **49**: 218. 1951.

FORBES, T. R. *Endocrinology* **52**: 236. 1953.

GREGERSEN, M. I.: *J. Lab. & Clin. Med.* **29**: 1266. 1944.

HARTMAN, C. G.: *Proc. Soc. Exper. Biol. & Med.* **48**: 221. 1941.

HARTMAN, C. G. AND G. W. CORNER: *Anat. Rec.* **98**: 539. 1947.

HASKINS, A. L. JR.: *J. Clin. Endocrinol.* **1**: 65. 1941.

HASKINS, A. L.: *Proc. Soc. Exper. Biol. & Med.* **73**: 439. 1950.

HOFFMAN, F. AND L. VON LAM: *Zentralbl. f. Gynak.* **70**: 1177. 1948.

HOOKER, C. W. AND T. R. FORBES: *Endocrinology* **41**: 158. 1947.

HOOKER, C. W. AND T. R. FORBES: *Endocrinology* **45**: 71. 1949.

LAZO-WASEM, E. A., G. M. NEHER, R. L. SHOGER AND M. X. ZARROW: *Endokrinol.* **31**: 166. 1954.

MELINKOFF, E.: *Am. J. Obst. & Gynecol.* **60**: 437. 1950.

NEHER, G. M. AND M. X. ZARROW: *Anat. Rec.* **108**: 68. 1950.

SCHULTZ, D. H.: *Am. J. Obst. & Gynecol.* **66**: 1260. 1953.

ZARROW, M. X. AND G. M. NEHER: *J. Clin. Endocrinol. & Metab.* **13**: 203. 1953.

ZARROW, M. X., R. L. SHOGER AND E. A. LAZO-WASEM: *J. Clin. Endocrinol. & Metab.* In Press, 1954.

LOCALIZATION OF PROTEIN-BOUND I^{131} IN THE THYROID GLAND OF THE MOUSE

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HIGH resolution autoradiography as been used to localize protein-bound I^{131} in the thyroid gland of the rat by Leblond and Gross (1948) and by Doniach and Pelc (1949). The theory and methods have been reviewed by Gross, Bogoroch, Nadler and Leblond (1951) and by Fitzgerald, Simmel, Weinstein and Martin (1953). A critical review of the results of autoradiographic studies on the thyroid has been published recently (Doniach, Howard and Pelc, 1953).

Two details which appeared to be unsettled were the site of the protein binding of iodine and the significance of the "ring" distribution observed for protein-bound I^{131} in the inactive gland. These problems and several others discussed by Leblond and Gross (1948) have now been investigated in the thyroid gland of the mouse.

MATERIALS AND METHODS

Male C3H mice 3 to 7 months of age weighing 25 to 35 gm. were used. They were fed a pelleted stock diet of moderately low iodine content (Wollman, Scow, Wagner and Morris, 1953.)

Procedure: Autoradiographs were obtained from thyroid glands excised at selected time intervals ranging from eleven seconds to one hour after radioiodide injection. To obtain thyroids as early as eleven seconds after radioiodide injection, the mice were anesthetized and thyroids exposed just prior to radioiodide injection.

When anesthetizing mice prior to radioiodide injection, Veterinary Nembutal (Abbott)[2] was used. The stock solution was diluted 1:10 with distilled water and 0.01 W+0.03 ml. was injected intraperitoneally where W was the mouse body weight in grams.

One-tenth ml. of a solution containing from 5 to 600 microcuries of carrier-free radioiodide[3] was injected either intravenously or intraperitoneally. Details of the treatment of each group of mice are summarized in Table 1.

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[1] National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare.

[2] This preparation contains 60 mg. of sodium pentobarbital per ml., and, in addition, 10% ethyl alcohol and 20% propylene glycol.

[3] The carrier-free radioiodide was obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee.

TABLE 1. SUMMARY OF EXPERIMENTAL DETAILS

Time interval I^{131} injection to thyroid excision	Number of mice	Anesthetic prior to I^{131}	Dose radioiodide microcuries	Radioiodide injection route	Fixative	Per cent dose protein bound
11 to 16 sec.	8	Nembutal	500 to 600	External jugular	Bouin	0.003 to 0.03[1]
11 sec.	2	Nembutal	500	External jugular	Carnoy	0.01, 0.03
2 min.	2	None	250	Tail vein	Bouin	0.2, 0.3
10 min.	3	Nembutal	25	Tail vein	Bouin and Carnoy	1.1 to 1.8
10 min.	4	None	25	Tail vein	Bouin	0.92 to 2.2
14 min.	3	Nembutal	40	Tail vein	Bouin	0.95 to 1.4
16 min.	3	None	25	Tail vein	Bouin	3.5
15 min.	4	None	500	Tail vein	Bouin	0.02 to 0.1[2]
60 min.	4	Nembutal	5 to 10	Intraperitoneal	Bouin	3.2 to 6.6[3]
60 min.	6	None	5	Intraperitoneal	Bouin and Carnoy	4.2 to 14.8[3]

[1] Protein-bound I^{131} was significantly greater for the longer time interval.
[2] Mice injected intraperitoneally with 15 micrograms iodide 15 minutes before radioiodide injection.
[3] The per cent of dose incorporated into protein bound I^{131} was generally less in mice given Nembutal than in unanesthetized mice.

Thyroids were excised from anesthetized mice. If mice were not anesthetized with nembutal prior to I^{131} injection, they were anesthetized with ether just before thyroid excision. To remove the thyroid, it was exposed, the trachea was cut simultaneously above and below the thyroid, and the thyroid and trachea were transferred to ice-cold fixative. Approximately one second was required for cutting the trachea and transferring the thyroid to fixative.

Fixation and preparation of tissue sections: Bouin's fluid was usually employed although some thyroids were fixed with Carnoy's fluid. The fixative and the first alcohol wash contained a little stable NaI to aid in washing out radioiodide. Tissues fixed in Bouin's were washed with three changes of 70% alcohol, and then carried through 80, 90 and 95% alcohol and finally absolute alcohol. Tissues fixed in Carnoy's were washed in several changes of absolute alcohol (Table 1). After washing out radioiodide for at least 16 hours using three changes of alcohol, the protein-bound I^{131} content of the tissue was measured using a well-type gamma counter to guide in the selection of exposure times (Table 1). Tissues were cleared in chloroform and embedded in paraffin. Serial sections were cut at 5 microns.

Autoradiographic technique: A technique similar to that of Evans (1947) was employed. The photographic film used was Kodak Autoradiographic Permeable Base Safety Stripping Film (Experimental). Small strips were allowed to spread on distilled water to approximately four times the original area and then mounted emulsion side up on clean microscope slides coated with gelatin. The thickness of emulsion layer of the original film was 5.6 microns,[4] and of that on the slides was approximately 1.5 microns. Tissue ribbons from the paraffin block were spread on water at 40° C., transferred to water at 20° C., and the floated onto the film emulsion and dried. After exposing the film from $\frac{1}{2}$ to 4 days in a dry light-tight box, the paraffin was removed by two rinses for 5 minutes each in xylene. After drying, the emulsion was developed using Dektol diluted 1:1. The slides were first immersed in developer for 2 minutes at 5° C. to insure sufficient time for the developer to penetrate the emulsion under the tissue sections (Goldschmidt-Clermont, 1953) and then for 1 to 2 minutes at 19° C. After fixation and washing in distilled water, the preparations were stained for 5 minutes in *Kernechtrot, Salz B* (Grübler) (Romeis, 1948), washed in water for 5 minutes, dehydrated, cleared in xylene and mounted in Fisher's Permount.

All conclusions are based on consistent findings in consecutive serial sections.

[4] We are indebted to W. H. Bowerman of the Eastman Kodak Company for the measurements of emulsion thickness.

RESULTS

When these autoradiographic procedures were used on thyroid glands from mice which had not been injected with radioiodide, or on thyroids from mice injected with radioiodide but in which the formation of protein-bound I^{131} had been prevented by prior injection of propylthiouracil, no blackening under the thyroid tissues was observed. In addition, the time interval required for a satisfactory film density correlated well with the amount of I^{131} in the thyroid gland. No evidence was found that the presence of tissue on the emulsion inhibited the development of the latent image. Background fog was just as dense under tissue sections as in the neighboring emulsion not covered by tissue.

In autoradiographs obtained from sections of thyroid glands excised as early as eleven to sixteen seconds after intravenous injection of radioiodide the image in the emulsion was largely in the form of narrow rings or incomplete rings under the edge of the colloid (Figs. 1–6). Although the image was localized almost entirely under the colloid, some was under the apical end of the epithelial cells.

Most of the protein-bound I^{131} responsible for the image was clearly localized in the colloid, but it was not possible to determine the localization of the protein-bound I^{131} responsible for the faint image under the cells in well-fixed follicles. However, in follicles in which the colloid had shrunken away from the epithelial cells (Figs. 3–6), the autoradiographic image was always associated with the edge of the colloid. The image under the cells was never darker than would be expected from background fog plus scattered radiation from the protein-bound I^{131} localized in the colloid.

To determine how long it took for the ring distribution to broaden into a uniform disk, autoradiographs were obtained from thyroid glands excised at approximately two minutes, 10 to 15 minutes and at 60 minutes after radioiodide injection. At the two latter time intervals, comparison was made of the results in anesthetized and unanesthetized mice.

In autoradiographs of thyroid sections from glands excised at approximately two minutes after I^{131} injection (Fig. 7) the image was distributed in a narrow ring under the edge of the colloid similar to the distribution in the eleven to sixteen second, anesthetized mice.

In autoradiographs obtained from thyroid glands excised from six of the seven anesthetized mice 10 to 15 minutes after radioiodide injection (Figs. 8, 10, 12) the image was still in the form of narrow rings or incomplete rings at the edge of the colloid, although in 1 of the 7 mice many rings were broadened almost to the point of being disks (Fig. 12). In contrast, in all the corresponding unanesthetized mice the autoradiographic image (Figs. 9, 11, 13) from most thyroid follicles was a uniform disk, although there were some broad rings and narrow rings. In addition in unanesthetized

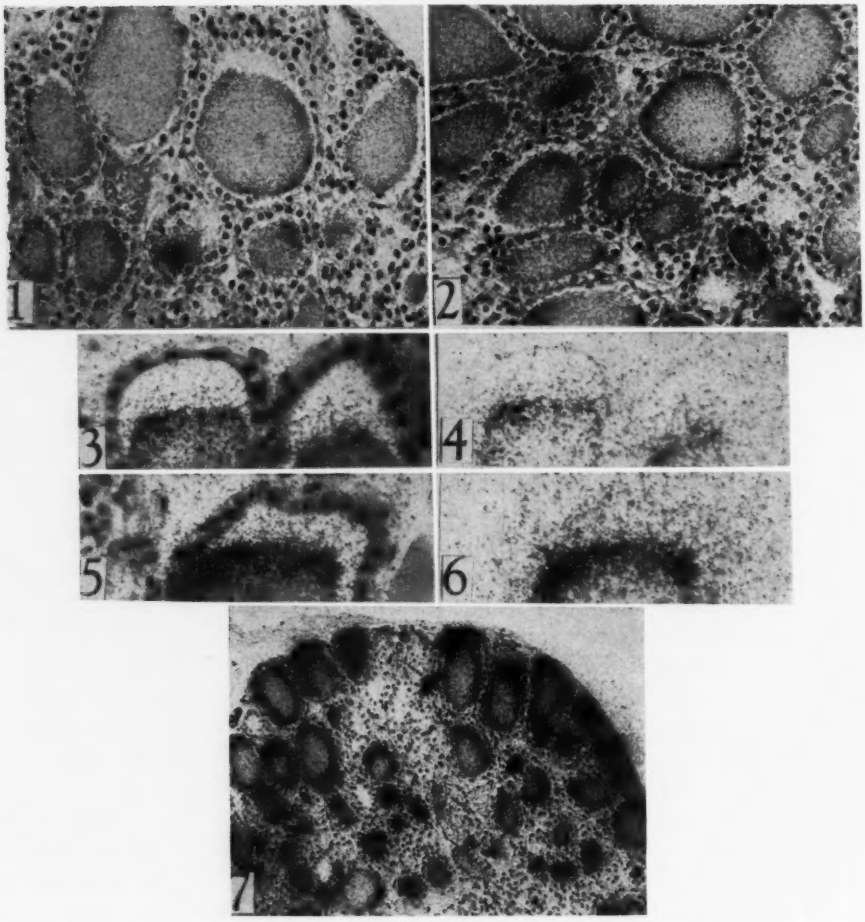

PLATE 1. AUTORADIOGRAPHS OF SECTIONS OF THYROID GLANDS OF MICE. *Kernechtrot, Salz B* (GRÜBLER) STAIN.

FIG. 1. Mouse anesthetized with nembutal. Thyroid excised 16 seconds after radioiodide injection. Bouin's fixation ×220.

FIG. 2. Mouse anesthetized with nembutal. Thyroid excised 15 seconds after radioiodide injection. Bouin's fixation ×220.

FIG. 3. Mouse anesthetized with nembutal. Thyroid excised 11.5 seconds after radioiodide injection. Carnoy's fixation ×430. Green filter.

FIG. 4. Same area as in Figure 3. Red filter.

FIG. 5. Mouse anesthetized with nembutal. Thyroid excised 11.5 seconds after radioiodide injection. Carnoy's fixation ×430. Green filter.

FIG. 6. Same area as in Figure 5. Red filter.

FIG. 7. Unanesthetized mouse. Thyroid excised 2.5 minutes after radioiodide injection. Bouin's fixation ×110.

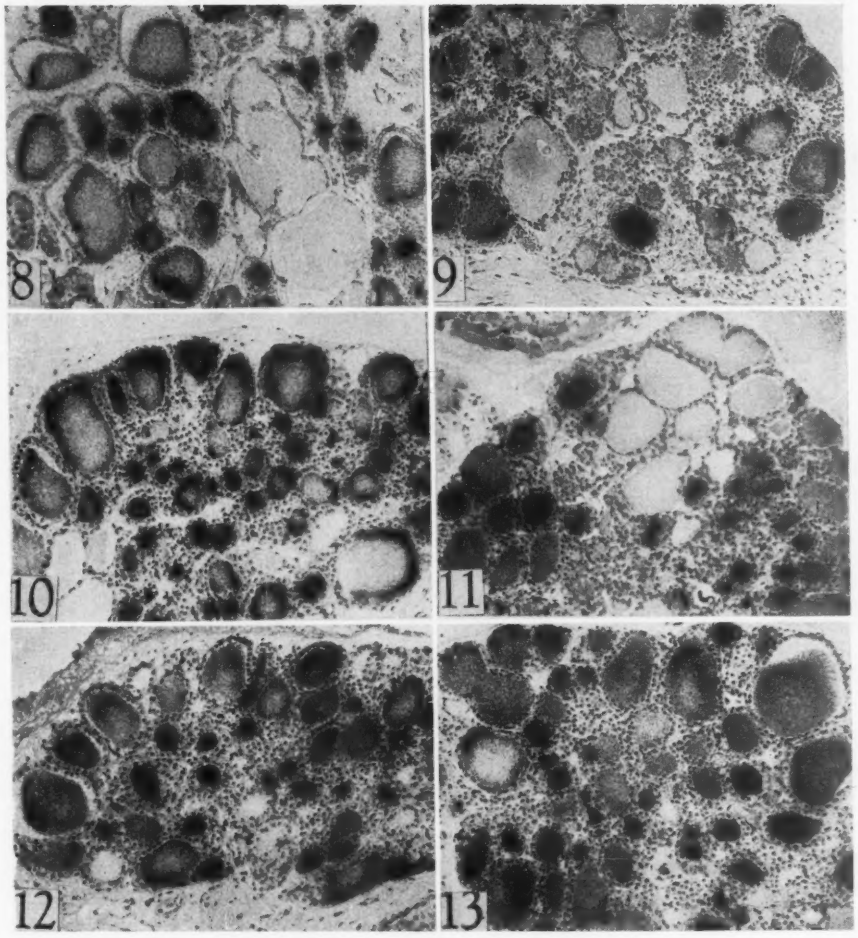

PLATE 2. AUTORADIOGRAPHS OF SECTIONS OF THYROID GLANDS OF MICE.
Kernechtrot, Salz B STAIN.

FIG. 8. Mouse anesthetized with nembutal. Thyroid excised ten minutes after radioiodide injection. Carnoy's fixation ×110.

FIG. 9. Unanesthetized mouse. Thyroid excised ten minutes after radioiodide injection. Bouin's fixation ×110.

FIG. 10. Mouse anesthetized with nembutal. Thyroid gland excised 14 minutes after radioiodide injection. Bouin's fixation ×110. Characteristic finding in anesthetized mice.

FIG. 11. Unanesthetized mouse. Thyroid excised 16 minutes after radioiodide injection. Bouin's fixation ×110. Note non-functional follicles.

FIG. 12. Mouse prepared as in Figure 10. Bouin's fixation ×110. Extreme case of broad rings observed in only one mouse.

FIG. 13. Unanesthetized mouse. Thyroid excised 16 minutes after radioiodide injection. Bouin's fixation ×110. Protein-bound I^{131} uniformly mixed in almost all follicles.

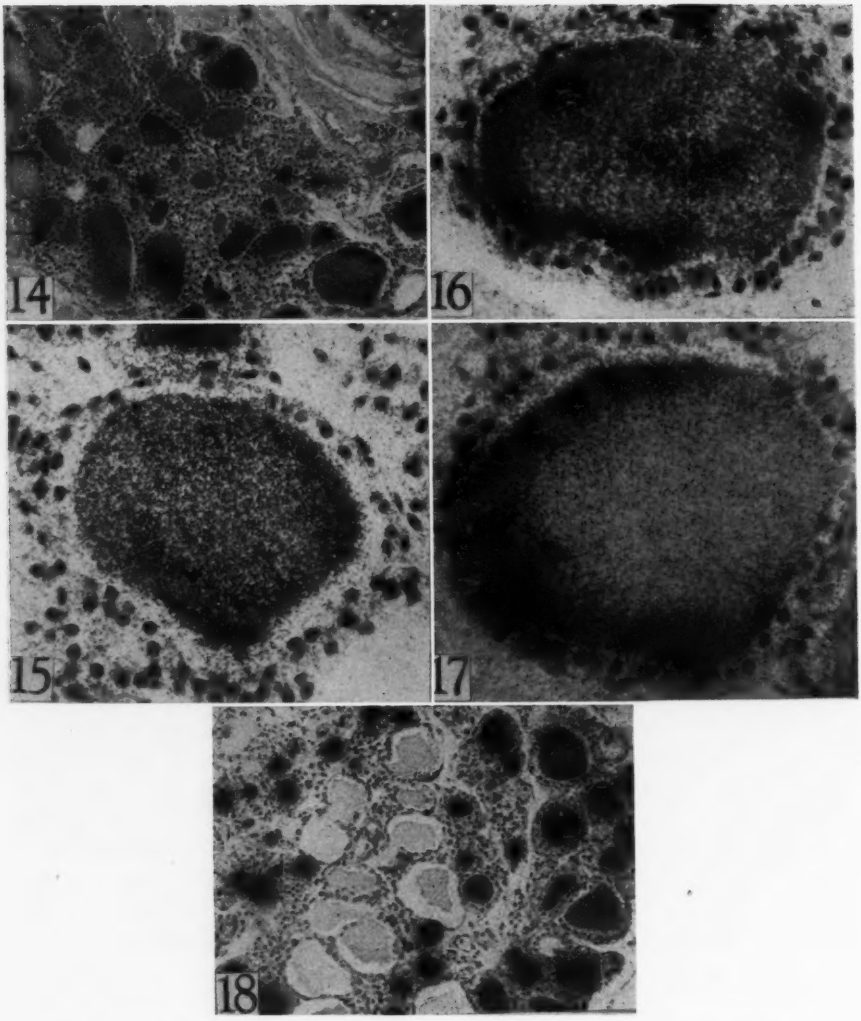

PLATE 3. AUTORADIOGRAPHS OF SECTIONS OF THYROID GLANDS OF MICE.
Kernechtrot, Salz B STAIN.

FIG. 14. Unanesthetized mouse injected with 15 μg. of stable iodide. Fifteen minutes later given intravenous injection of radioiodide. Thyroid excised fifteen minutes after radioiodide injection. Bouin's fixation $\times 110$.

FIG. 15. Single follicle of mouse prepared as in Figure 14. $\times 430$. Note protein-bound I^{131} is localized in colloid.

FIG. 16. Single follicle from thyroid of a mouse prepared as in Figure 13. $\times 430$. Note that non-uniform distribution of protein-bound I^{131} can occur in unanesthetized mouse.

FIG. 17. Single follicle from thyroid of a mouse prepared as in Figure 10. $\times 430$. Note non-uniform ring and localization of protein-bound I^{131} to within colloid.

FIG. 18. Unanesthetized mouse. Thyroid excised one hour after radioiodide injection. Bouin's fixation $\times 110$. Note non-functional follicles.

mice a follicle was occasionally observed in which the image suggested that the protein-bound I^{131} had been formed at a small segment of the circumference and had diffused from there gradually into the rest of the colloid, but had not yet uniformly mixed.

In mice injected with 15 μg. of stable iodide 15 minutes before radioiodide injection the I^{131} uptake was only about 3% of that of control mice not given stable iodide (Table 1). However, the autoradiographic image (Figs. 14, 15) was not significantly different from that in the control mice.

Autoradiographs from thyroid glands excised 1 hour after radioiodide injection of anesthetized mice yielded images of broad rings and narrow rings although there were many uniform disks. On the other hand, autoradiographs from the corresponding unanesthetized mice (Fig. 18) yielded uniform disk distributions except for a rare persistent narrow ring or incomplete ring.

Incomplete narrow ring autoradiographs were observed in thyroid sections from some glands excised at each of the time intervals after radioiodide injection (Figs. 1, 7, 10). In unanesthetized mice this observation was relatively rare at time intervals at or after 10 minutes because so few follicles exhibited rings. In anesthetized mice or in the two minute group (Fig. 7) when incomplete rings appeared they frequently occurred in follicles at the periphery of the section and in these the open side of the ring was almost always localized to the part of the colloid of the follicle near the interior of the gland.

The width of the narrow rings was with one exception approximately the same in glands from anesthetized mice whether thyroids were excised at 15 seconds or 15 minutes after radioiodide injection.

Many follicles in otherwise normal glands gave no autoradiographic image (Figs. 8, 9, 10, 11, 18). Most of these follicles contained a foamy or granular colloid; in some follicles the colloid contained a single centrally located vacuole, and in some the colloid appeared normal when stained with *Kernechtrot, Salz B*. In almost all of these follicles some of the epithelial cells were ciliated.

DISCUSSION

These autoradiographic studies indicate that all detected protein-bound I^{131} was localized in the colloid. This localization occurred not only in unanesthetized mice one hour after radioiodide injection, but in anesthetized mice as early as 11 seconds after radioiodide injection. The shortest time interval studied is really less than the above 11 seconds since several seconds are probably required for the radioiodide to travel from the injection site (external jugular vein) to the thyroid gland.

The autoradiographic technique used appears adequate to detect pro-

tein-bound I^{131} it if were localized in the fixed cells. The cells did not appear to inhibit the development of the latent image under the cells. However, it appears advisable to retain reservations with respect to the conclusions drawn from the above observations on the localization of protein-bound I^{131} to the colloid for the following reasons: (1) It is difficult to exclude the possibility that the protein-bound I^{131} was synthesized in the epithelial cells and secreted into the colloid during the time interval between thyroid excision and fixation. However, this process appears improbable since the bare thyroid was in direct contact with ice-cold fixative which must have rapidly cooled the tissue, promptly slowing down any secretory process. In addition, Bouin's fluid penetrates tissue rapidly (Medawar, 1941) and would need to penetrate only approximately 10 μ to stop physiological activity in the outermost follicles. Protein-bound I^{131} was not localized in cells of either exterior or interior follicles.

(2) It is possible that protein-bound I^{131} might be extracted from the epithelial cells by the fixative. This possibility might be minimized by comparing results using a wide variety of fixatives.

(3) When colloid shrinks, part of the apical end of the epithelial cells, especially the brush border described by Braunsteiner, Fellinger and Pakesch (1953), might remain with the colloid. In such a case, if some protein-bound I^{131} were localized in the brush border, the protein-bound I^{131} would misleadingly appear to be localized in a completely extracellular structure. Since the width of the narrow rings was so much larger than the thickness of brush borders, this possibility could not account for all the protein-bound I^{131} in narrow rings.

(4) A high concentration of intracellular protein-bound I^{131} might be localized to such a small volume of the epithelial cells that the total amount of protein-bound I^{131} would not be sufficient to yield a detectable autoradiograph.

With the above reservations in mind, the following conclusions are drawn: These observations support the suggestion of Doniach, Howard and Pelc (1953) that the iodination process normally occurs in the colloid and not in the cytoplasm of the epithelial cells. This conclusion, based on observations on the mouse, is quite different from that of Leblond and Gross (1948) for the rat. The observations also suggest that there is no reason to believe that the appearance of protein-bound I^{131} in the colloid indicates the secretion of thyroglobulin. In contrast, they suggest that non-iodinated or only partially iodinated protein is already present in the colloid waiting to be iodinated. The present results do not exclude the possibility that colloid droplets reported to be present in some thyroid cells may be partially iodinated prior to extrusion into the colloid. These results have no immediate bearing on the site of localization of the oxida-

tive step which has been presumed to occur prior to the iodination process. It is an interesting correlation, however, that radioiodide appears to be localized largely in the colloid of the thyroid both in the rat (Pitt-Rivers and Trotter, 1953) and in the mouse (Wollman and Wodinsky, unpublished observations) when binding is blocked by propylthiouracil.

Although the present results make it appear probable that for the most part the iodination of thyroglobulin occurs in the colloid in the normal mouse it is not yet possible to conclude that no iodination occurs in the thyroid epithelial cell. Major evidence for intracellular iodination at present comes from the reports that organically bound iodine was found to occur apparently prior to the appearance of colloid in the thyroid gland of the fetal pig (Rankin, 1941) and the fetal calf (Koneff, Nichols, Wolff and Chaikoff, 1949). Additional evidence is the report that protein-bound I^{131} was localized in the epithelial cells in rats fed a high iodine diet (Leblond and Gross, 1948).[5] In some preliminary studies on normal rats fed the stock diet we found that as in the mouse the narrow ring autoradiographic image was localized almost entirely under the colloid in thyroids excised 30 seconds after radioiodide injection. In view of the above remarks it appears that the diiodotyrosine and thyroxine found by Rerabek (1952) to be localized in the non-particulate fraction of the cytoplasm and possibly the colloid of the thyroid gland of the pig was probably localized in the colloid.

Although the protein-bound I^{131} was found to be localized in the colloid, a time interval of over 15 minutes was required for uniform mixing in many of the follicles, and over an hour was required in a few of the follicles. This implies that colloid is normally viscous. The observation that the mixing of protein-bound I^{131} occurs more rapidly in some follicles than in others suggests that the viscosity of the colloid or that the shape or size of the thyroglobulin molecules differs in different follicles. In addition in preliminary studies it was found that narrow ring autoradiographs persisted in almost all follicles in two normal young rats in which the I^{131} uptake in one hour by the thyroid gland was 10% of the dose. These results suggest that the use of specific activity studies in the rat (Taurog and Chaikoff, 1947) and mouse to determine the rates of synthesis of thyroxine may not be expected to yield results of quantitative significance unless parallel autoradiographic studies are made which show that in the animals used there is rapid, uniform mixing of the colloid.

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[5] It may be that this is not good evidence since in a recent personal communication Dr. Leblond says that measurements of radioautographic densities on profile sections made it possible to assign most "ring" reactions to the peripheral portion of the colloid rather than to the cell apex (N. J. Nadler and C. P. Leblond, unpublished).

Anesthetization with a Veterinary Nembutal solution was associated with a marked apparent increase in the viscosity of the colloid. No study has been made to ascertain whether the effect was due to the sodium pentobarbital itself, to other substances present in the preparation, or whether the effect was an indirect one. However, this observation suggests that caution should be taken in evaluating the qualitative observations of the viscosity of the colloid in thyroid follicles of rats anesthetized with nembutal (de Robertis, 1941).

Autoradiographic images indicating incomplete ring distributions of protein-bound I^{131} in the colloid of the mouse thyroid were frequently observed. In general, in peripheral follicles the open end of the ring faced the interior of the gland. This is opposite to the situation in the rat in which the open end of the ring faced the periphery of the gland (Leblond and Gross, 1948; Wollman and Wodinsky, unpublished observations). Possible explanations for the incomplete rings are that the capillary net around some follicles is incomplete or not uniformly patent or that some of the epithelial cells are not functioning.

Autoradiographic studies of protein-bound I^{131} in thyroid glands excised from animals at very short time intervals after radioiodide injection ought to be useful in deciding whether I^{131} is accumulated in all follicles continuously or in a cyclic fashion. It is not unreasonable to expect cyclic activity with a short period since the blood vessels in many tissues exhibit vasomotor activity. Of course, in order to observe this effect the time interval between radioiodide injection and thyroid excision must be short compared with the period of the cycle. Unfortunately, the mouse does not appear to be a suitable animal for such observations because the mouse thyroid possesses two types of follicles (Dunn, 1944) and one of these is ultimobranchial in origin and does not accumulate I^{131} (Gorbman, 1947). Although it was usually easy to distinguish the two types of follicles, occasionally it was not possible to tell whether or not a particular follicle was ultimobranchial in origin. On the other hand, in anesthetized rats all follicles fixed I^{131} in the colloid as early as 30 seconds after radioiodide injection (Wollman and Wodinsky, unpublished) in agreement with earlier observations by Leblond and Gross (1948).

The dependence of the concentration of protein-bound I^{131} in a functional follicle on the size of the follicle seemed to be in qualitative agreement with the results of Nadler, Leblond and Bogoroch (1954) in rats. In general, the concentration of protein-bound I^{131} in the follicular colloid decreased as the size of the follicle increased although there were some obvious exceptions. Interpretation of this qualitative relationship and of the report by Nadler, Leblond and Bogoroch (1954) that uptake of I^{131} by a follicle in a rat thyroid was proportional to the surface area of the follicle

may be aided by a suggestion implicit in the results of some recent studies of the iodide concentrating ability of thyroid tissue. These studies suggest that the rate of blood flow to the thyroid gland strongly limits the uptake of I^{131} (Wollman and Scow, 1953, and unpublished observations in the rat). Analysis of the consequences of these studies (Wollman, 1954) suggests that in this special case the uptake of I^{131} by a follicle may be described most appropriately as being proportional to the product of the extraction ratio of the follicle and the volume of blood flowing to the follicle per unit time. The dependence of these factors on follicle size is unknown at present. Direct observation of the dependence of the rate of volume flow of blood on follicle size *in vivo* might allow evaluation of the relative contribution of each of these factors to the variation of I^{131} uptake with follicle dimensions.

SUMMARY

Autoradiographic studies were made of the localization of protein-bound I^{131} in the thyroid gland of C3H mice between eleven seconds and one hour after radioiodide injection. The protein-bound I^{131} responsible for the autoradiographic image always appeared to be localized in the colloid. This suggested that the iodination of the thyroglobulin occurred in the colloid. At two minutes and earlier the protein-bound I^{131} in thyroid sections appeared to be localized in a narrow ring at the edge of the colloid. By ten minutes the ring distribution had become broad rings or uniform disks in most of the follicles, but the variation from follicle to follicle within a single gland suggested that the colloid was of variable viscosity from one follicle to another. Anesthesia with Veterinary Nembutal appeared to increase significantly the time interval required for rings to become disks or to increase the viscosity of the colloid. Occasionally, follicles yielded incomplete ring autoradiographs indicating non-uniform function. Some follicles did not accumulate detectable amounts of I^{131}.

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REFERENCES

BRAUNSTEINER, H., K. FELLINGER AND F. PAKESCH: *Endocrinology* **53**: 123. 1953.
DONIACH, I., A. HOWARD AND S. R. PELC: *Progress in Biophysics* **3**: 1. 1953.
DONIACH, I. AND S. R. PELC: *Proc. Roy. Soc. Med.* **42**: 957. 1949.
DUNN, T. B.: *J. Nat. Cancer Inst.* **4**: 555. 1944.
EVANS, T. C.: *Proc. Soc. Exper. Biol. and Med.* **64**: 313. 1947.
FITZGERALD, P. J., E. SIMMEL, J. WEINSTEIN AND C. MARTIN: *Lab. Invest.* **2**: 181. 1953.
GOLDSCHMIDT-CLERMONT, Y.: *Ann. Rev. Nuclear Science* **3**: 141. 1953.

GORBMAN, A.: *Anat. Rec.* **98**: 93. 1947.
GROSS, J., R. BOGOROCH, N. J. NADLER AND C. P. LEBLOND: *Am. J. Roent.* **65**: 420. 1951.
KONEFF, A. A., C. W. NICHOLS, JR., J. WOLFF AND I. L. CHAIKOFF: *Endocrinology* **45**: 242. 1949.
LEBLOND, C. P. AND J. GROSS: *Endocrinology* **43**: 306. 1948.
MEDAWAR, P. B.: *J. Roy. Microscop. Soc.* **61**: 46. 1941.
NADLER, N. J., C. P. LEBLOND AND R. BOGOROCH: *Endocrinology* **54**: 154. 1954.
PITT-RIVERS, R. AND W. R. TROTTER: *Lancet* **265**: 918. 1953.
RANKIN, R. M.: *Anat. Rec.* **80**: 123. 1941.
RERABEK, J.: *Biochim. Biophys. Acta* **8**: 389. 1952.
DE ROBERTIS, E.: *Anat. Rec.* **80**: 219. 1941.
ROMEIS, B.: Mikroscopische Technik. Leibniz Verlag, München. 1948.
TAUROG, A. AND I. L. CHAIKOFF: *J. Biol. Chem.* **169**: 49. 1947.
WOLLMAN, S. H.: *Endocrinology* **54**: 35. 1954.
WOLLMAN, S. H. AND R. O. SCOW: *Endocrinology* **53**: 332. 1953.
WOLLMAN, S. H., R. O. SCOW, B. WAGNER AND H. P. MORRIS: *J. Nat. Cancer. Inst.* **13**: 785. 1953.

ROLE OF POTASSIUM IN THE PROTEIN-CATABOLIC EFFECT OF CORTISONE AND ACTH[1]

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SEVERAL recent studies have been concerned with the possible interrelationship of changes of potassium metabolism and of protein and carbohydrate metabolism under the influence of adrenocortical hormones. Ingestion of large amounts of potassium chloride inhibits the diabetes enhancing effect of ACTH in partially depancreatized diabetic rats (Glafkides, Bennett, and George, 1952). A similar effect of potassium chloride has been observed in the ACTH or cortisone treated diabetic human in as much as the insulin requirements were not increased by cortisone or ACTH (Kinsell, 1953). Inhibition by KCl of the protein catabolic effects produced by ACTH in the intact rat has been reported (Whitney and Bennett, 1952). However, the increase of urinary nitrogen excretion produced in patients with rheumatoid arthritis by ACTH was not prevented or ameliorated by potassium salts (Bennett, Liddle, and Bentnick, 1953). Also, in diabetics KCl administration failed to alter the nitrogen excretion increased by treatment with cortisone or ACTH (Kinsell *et al.*, 1954). Since we were not aware of any reports concerning the influence of KCl on alterations of the protein metabolism produced in rats by cortisone, it was considered of interest to determine whether large doses of KCl would inhibit or diminish the increase in urinary nitrogen excretion which occurs in cortisone treated tube fed rats. The urinary nitrogen excretion of rats treated with KCl and ACTH was also determined.

METHODS

Forty-three male rats, descendants of the Wistar strain (obtained from Carworth Farms), and weighing between 200 and 250 gm. were divided into the following groups.

Group I: 11 animals were treated with 300 mg. of KCL twice daily. Group II: 12 animals were treated with 2.5 mg. of cortisone acetate twice daily. Group III: 10 animals were treated with 300 mg. of KCL twice daily and 2.5 mg. of cortisone acetate twice

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daily. Group IV: 5 animals were treated with 5 units of ACTH twice daily. Group V: 5 animals were treated with 5 units of ACTH twice daily and 300 mg. of KCL twice daily.

The KCL was added to the diet; the cortisone or ACTH was injected subcutaneously at the same time twice daily. The control and treatment periods were of equal length, each seven days long. The diet used, the methods of tube feeding and housing the animals, and the methods of urine collection and urinary nitrogen determination have been described previously (Rupp. Paschkis, and Cantarow, 1949).

RESULTS

The pertinent data are summarized in Table 1.

The addition of 600 mg. of KCl to the daily food intake of tube-fed rats did not alter the urinary nitrogen excretion of the animals (Group I). Cortisone acetate in the dose used produced the expected increase of urinary excretion (Group II). This increase in urinary nitrogen excretion produced by cortisone was not altered by large doses of KCl (Group III). The increase in urinary nitrogen excretion produced by ACTH (Group IV) was not altered by KCl (Group V).

The weight gain of the tube-fed rats was not influenced by KCl (Group I), nor was the weight loss induced by cortisone (Group II), or by ACTH (Group IV), altered by the addition of KCl (Groups III and V).

DISCUSSION

Large doses of KCl did not influence the protein metabolism of tube-fed rats as gauged by nitrogen excretion, nor did they alter the protein catabolic effects induced by cortisone or ACTH in tube-fed rats. These findings are similar to those reported for man (Bennett, Liddle, and Bentnick, 1953; Kinsell et al., 1954). In the rat, no previous study has been made with cortisone, however the results of the experiment with ACTH and KCL (Group V) differ from those obtained in the rat by Whitney and Bennett, 1952). In our experiment large doses of KCL did not alter the protein catabolic effects of ACTH whereas Whitney and Bennett reported that KCL inhibited the catabolic effect of ACTH. The reason for these differ-

TABLE 1. INFLUENCE OF KCL ON THE DAILY URINARY N EXCRETION OF RATS TREATED WITH CORTISONE ACETATE OR CORTICOTROPHIN

Group	No. of rats	Mg./24 hours		Urinary *N* Excretion, mg./24 hrs.		Diff.	"p" of diff.	Wt. change, gm./7 days	
		KCl	Cortisone ac.	Control period	Treatment period			Control	Treated
I	11	600	0	226 ± 10.65*	234 ± 10.51	+ 8	<0.5	+8	+ 8
II	12	0	5	220 ± 9.21	307 ± 13.81	+87	<0.001	+7	−12
III	10	600	5	220 ± 11.69	305 ± 13.86	+85	<0.001	+8	−10
		KCl mg./24 hrs.	ACTH units/24 hrs.						
IV	5	0	10	227 ± 6.40	263 ± 6.48	+36	<0.001	+6	− 3
V	5	600	10	236 ± 4.10	277 ± 16.54	+41	<0.001	+9	− 2

* Standard deviation.

ences is not immediately apparent. It is improbable that the different strains of rats used would account for the difference in results. Whitney used rats of the Long Evans strain whereas rats of the Wistar strain were used in our experiment. The dosage of ACTH used cannot be compared since the amounts employed are expressed in different units. It is unlikely that the difference would be the result of the different methods used for feeding. In our experiments the rats were fed twice daily, while the animals used by Whitney and Bennett were trained to eat a constant amount of food each day (Bennett and Li, 1947). The protein (2 gm. daily) fed the rats in our experiment was less than the protein (3.2 gm. daily) fed the animals used by Whitney and Bennett (Whitney and Bennett, 1952). The possibility that this difference in protein intake would alter the inhibiting influence of KCL on the protein catabolic effect of ACTH is an interesting one, which has not been studied.

SUMMARY

Large doses of KCL did not influence the increase in urinary nitrogen excretion nor the weight loss induced by cortisone or ACTH in tube fed rats.

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REFERENCES

Bennett, L. L. and C. H. Li: *Am. J. Physiol.* **150:** 400. 1947.

Bennett, L. L., G. W. Liddle and R. G. Bentnick: *J. Clin. Endocrinol. and Metab.* **13:** 392. 1953.

Glafkides, C. M., L. L. Bennett and R. George: *Endocrinology* **50:** 684. 1952.

Kinsell, L. W.: *Lancet* **73:** 222. 1953.

Kinsell, L. W., G. D. Michaels, S. Margon, J. W. Partridge, L. Boling and H. E. Balch: *J. Clin. Endocrinol. and Metab.* **14:** 167. 1954.

Rupp, J., K. E. Paschkis and A. Cantarow: *Endocrinology* **44:** 449. 1949.

Whitney, J. E. and L. L. Bennett: *Endocrinology* **50:** 657. 1952.

IRRADIATION AND ADRENAL STEROIDOGENESIS:[1]

STEROID TRANSFORMATIONS BY IRRADIATED ISOLATED PERFUSED CALF ADRENALS

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INTRODUCTION

PREVIOUS studies by Ungar, Rosenfeld, Dorfman and Pincus (1954) have demonstrated that exposure of the isolated, ACTH-stimulated calf adrenal to moderate doses of gamma radiation during perfusion results in a significantly decreased secretion of hydrocortisone, corticosterone, and unidentified Porter-Silber chromogens. The object of the present investigation was to determine whether the diminished secretion of these metabolically important corticoids was attributable to an impaired biosynthesis of the steroid nucleus, a defect on the 11β-, 17α-, or 21-hydroxylation activity, or to a combination of these possible individual defects.

In order to ascertain the effect of ionizing radiation on specific adrenocortical biosynthetic enzymatic systems, various steroid substrates were perfused without ACTH through control and irradiated calf adrenal glands and the major transformation products isolated and determined. The substrate conversions investigated have been previously demonstrated by the group at the Worcester Foundation, and involve 11β-, 17α-, and 21-hydroxylations, and the oxidation of the Δ^5-3β-hydroxyl group to the Δ^4-3-ketone group (Hechter *et al.*, 1951).

MATERIALS AND METHODS

Perfusion of calf adrenal glands. Fresh calf blood and adrenal glands were obtained from a local slaughterhouse on the day of perfusion. The cannulation and perfusion procedures were the same as those employed by Hechter (1948). Prior to adrenal passage, six liters of homologous citrated blood were perfused through isolated rat livers to re-

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[2] Opinions and assertions are those of the authors and do not necessarily reflect those of the U. S. Naval Service.

move vasoconstrictor and other undesirable substances. The various steroid substrates, viz. DOC, Substance S, progesterone, 21-desoxy E, and dehydroepiandrosterone were dissolved in minimal volumes of acetone to which 20 ml. of propylene glycol were added, and the acetone removed at 50° C. with a stream of nitrogen. Irradiated and contralateral control groups of four adrenals were perfused for one hour with a liter of liver-perfused blood which was discarded. The glands were then perfused for two additional hours with a second liter of prepared blood to which an aliquot of the steroid-propylene glycol solution had been added dropwise and with thorough mixing. Individual recovery experiments were conducted for each study by stimultaneously oxygenating and circulating a liter of the same steroid-blood mixture in a third perfusion apparatus without mounted organs.

Irradiation. In each experiment, a group of four glands was perfused while being exposed to 4 π gamma radiation from Co^{60} in the Radiocobalt Large Animal Irradiator of the Naval Medical Research Institute (Draeger *et al.*, 1953). The primary beam had an energy of 1.23 Mev. (with less than 10% softer scattered radiation). The dose rate ranged from 14 to 17 roentgens per minute in air as measured on a Victoreen rate meter with a 4 mm. lucite cap. The average dose rate to the glands was approximately in the same range.

Blood extraction and analysis. The ethyl acetate procedure for extracting the adrenal effluents (described in the succeeding article) was modified by allowing the blood to come to room temperature before extraction, and by increasing the number and period of extractions. Duplicate aliquots of the prepared blood extract were chromotographed on paper in a suitable system. The chromatographic zones of the substrate and the major conversion product were located, eluted, and quantitatively determined by methods previously described. Depending on the steroid, the Porter-Silber (1950), phosphomolybdic (Burstein, 1953), and Zimmermann (Holtorff and Koch, 1940) reactions as well as ultraviolet absorption at 240 mμ were applied. The major conversion product was isolated and identified by infrared analysis and melting point.

RESULTS

Table 1 is a summary of the radiation doses and gland weights of eleven experiments in which various steroid substrates were perfused through irradiated and control groups of four calf adrenals. The analytical results are summarized in Table 2. The original amount of substrate added to the perfusion blood is listed in Column 1. The total dose in roentgens of Co^{60} gamma radiation to which the irradiated groups were exposed during the 3-hour perfusion period is given in Column 2. Column 3 shows the amount in mg. of the major conversion product formed, as determined by the appropriate assay method on the eluate of the paper chromatographic zone. Column 4 presents the percentage of substrate converted by the control and irradiated glands, and column 5, the percentage decrease in conversion due to irradiation.

Conversion of Substance S to hydrocortisone. In three separate experiments, the conversion of Substance S to hydrocortisone by 11β-hydroxylation was decreased 75, 34 and 54%, respectively, by irradiation. The hydrocortisone zones were quantitatively determined by the Porter-Silber reaction. A

TABLE 1. IRRADIATION DOSES AND ADRENAL WEIGHT DATA OF THE STEROID TRANSFORMATION EXPERIMENTS

Steroid substrate perfused	Weight of 4 glands		Irradiation Data		
	Irradiated gm.	Control gm.	Dose rate* r/min.	Exposure time min.	Total dose r
Substance S	11.95	11.99	14.4	164	2,363
	14.78	14.96	14.4	168	3,419
	15.74	15.26	14.4	172	2,477
DOC	11.98	12.08	14.4	169	2,436
	13.73	13.19	14.4	162	2,334
Progesterone	12.90	12.92	14.0	173	2,422
	13.78	13.64	14.0	183	2,562
21-desoxy E	12.44	12.48	14.4	171	2,462
	13.19	13.20	14.0	178	2,492
dehydroepi-androsterone	14.58	14.60	14.0	166	2,324
	14.60	14.59	14.0	179	2,506

* As measured in air on a Victoreen rate meter.

sample of hydrocortisone, m.p. 204-210°, was crystallized from an aliquot of this zone and shown to be identical to an authentic sample by infrared analysis.

Conversion of desoxycorticosterone to corticosterone. Irradiation caused de-

TABLE 2. TRANSFORMATION OF STEROID SUBSTRATES PERFUSED THROUGH CONTROL AND Co^{60} IRRADIATED CALF ADRENAL GLANDS*

Steroid substrate and major transformation product	(1) Substrate added to the blood mg.	(2) Total radiation dose r	(3) Major product formed		(4) Substrate conversion		(5) Decreased conversion due to irradiation %	Specific reactions
			Control mg.	Irrad. mg.	Control %	Irrad. %		
Δ^4-pregnene-17α, 21-diol-3, 20-dione (Substance S)	62	2,363	5.8	1.5	9.3	2.3	−75	11 β-hydroxylation
↓	25	2,419	4.3	2.8	17.2	11.3	−34	
hydrocortisone	25	2,477	6.7	3.2	27.5	12.7	−54	
Δ^4-pregnene-21-ol-3, 20-dione (DOC)	100	2,436	32.4	21.5	32.4	21.5	−35	11 β-hydroxylation
↓	100	2,334	29.4	13.2	29.4	13.2	−55	
corticosterone								
progesterone	48	2,422	4.1	2.5	8.4	5.3	−37	11 β, 17α, and 21-hydroxylations
↓	48	2,562	4.3	4.5	8.9	9.5	+ 7	
hydrocortisone								
Δ^4-pregnene-17α-ol-3, 11, 20-trione (21-desoxycortisone)	25	2,462	4.1	3.2	16.2	12.6	−22	21-hydroxylation
↓	24	2,492	3.8	3.0	15.6	12.7	−19	
cortisone								
Δ^5-androstene-3 β-ol-17-one	50	2,324	19.7	10.3	39.4	20.5	−48	11 β-hydroxylation and Δ^5-3 β-OH → Δ^4-3-ketone
↓	50	2,506	13.4	10.6	26.7	21.2	−21	
11 β-hydroxy-Δ^4-androstene-3, 17-dione								

* Not stimulated with ACTH

creases of 34 and 55% in the conversion of desoxycorticosterone to corticosterone by 11β-hydroxylation, in two experiments. The corticosterone zones were quantitatively determined by the phosphomolybdic acid method. A sample of corticosterone, m.p. 178–182°, was obtained from an aliquot of this zone and was shown to be identical to an authentic sample by infrared analysis.

Conversion of progesterone to hydrocortisone. As a result of 11β-, 17α-, and 21-hydroxylations, hydrocortisone was the major conversion product of progesterone, although minor zones were observed, as in the other experiments. Exposure to Co^{60} gamma radiation induced a 37% decrease in the amount of progesterone converted to hydrocortisone in one study, but in another, no diminution was observed. In the latter experiment, however, dissection of the adrenals at the end of the 3-hour perfusion period indicated that at least one and probably two of the control glands were improperly perfused. The hydrocortisone zones were quantitatively determined by the Porter-Silber reaction. A sample of crystalline hydrocortisone, m.p. 205–211°, was obtained from the hydrocortisone zone and was identical to an authentic sample by infrared analysis.

Conversion of 21-desoxycortisone to cortisone. In two experiments, decreases of 22 and 19% were noted in the ability of the irradiated glands to convert 21-desoxycortisone to cortisone by 21-hydroxylation. The cortisone zones were quantitatively determined by the Porter-Silber reaction. The identity of the cortisone obtained from the cortisone zone was confirmed by infrared analysis.

Conversion of dehydroepiandrosterone to 11β-hydroxy-Δ^4-androstene-3,17-dione. The irradiated glands exhibited decreases of 48 and 21% in their ability to 11β-hydroxylate and to convert the Δ^5-3β-hydroxyl group to the Δ^4-3-ketone group. The 11β-hydroxy-Δ^4-androstene-3,17-dione zone was quantitatively determined by the Zimmermann reaction and by ultraviolet absorption at 240 mu. The identity of the 11β-hydroxy-Δ^4-androstene-3,17-dione was confirmed by infrared analysis.

DISCUSSION

Various model steroids were added to homologous blood containing no added ACTH and perfused through irradiated and control calf adrenals to ascertain by specific enzymatic conversion studies whether the reduction in corticoid output induced by irradiation (Ungar, Rosenfeld, Dorfman and Pincus, 1955) was attributable to a specific or general biosynthetic defect in the steroidogenic mechanism. The results demonstrated that irradiation caused a significant decrease in the transformation of the steroid substrates to products involving 11β-, 17α-, and 21-hydroxylations as well as the oxidation of the Δ^5-3β-hydroxyl group to the

Δ^4-3-ketone group. The percentage decrease in the conversion products paralleled, both in range and variability, the percentage decrease in corticoid output which was found in the ACTH-stimulation studies. Inasmuch as irradiation causes reductions of the same order of magnitude in the over-all biosynthetic and specific enzymatic activities, it would appear that its effect is generalized and exerted on all the steroidogenic enzymes rather than on any particular one. It is generally believed that the primary effect of ionizing radiations is on the cellular water and protein molecules. Irradiation putatively causes a denaturation of the cellular proteins and the formation of hydrogen peroxide and other oxidizing agents toxic to enzymes. Accordingly, one might expect all the protein enzyme systems involved in the steroidogenic mechanism to be susceptible to inactivation by ionizing radiation. The wide range of response to the same dose of radiation exhibited by different groups of glands suggests that the adrenal may possess some intrinsic and variable property which may alter its radiosensitivity. It should be added that the glands were obtained from physically and psychically stressed slaughterhouse animals, and, consequently might be in various states of responsivity both to ACTH and irradiation.

The main objective of these investigations and those reported in the preceding paper (Ungar *et al.*, 1954) was the study of steroidogenic function in perfused adrenals, isolated from the metabolic influences of other tissues and exposed *in vitro* to gamma radiation in order to investigate the primary effects of ionizing radiation on corticosteroidogenic enzymatic systems. Similar steroidogenic studies will be conducted with calf adrenals excised from whole-body irradiated animals. The latter systemic approach should provide information concerning the primary as well as secondary effects of irradiation on adrenocortical activity, and, in contrast to the studies already presented, would be complicated by the following *in vivo* factors: (1) adrenocortical pseudo-recovery, i.e. regeneration from uninjured cortical cells, (2) toxic products from nonadrenal tissues, and (3) removal of toxic products by excretion and detoxification.

SUMMARY

Exposure of the isolated calf adrenal to moderate doses of gamma radiation (Co^{60}) during perfusion of various steroid substrates caused a significant reduction in the ability of the gland to carry out 11β-, 17α, and 21-hydroxylations as well as the oxidation of the Δ^5-3β-hydroxyl group to the Δ^4-3-ketone group. The decrease in the specific enzymatic activities parallel the previously observed decrease in corticosteroid output by ACTH-stimulated irradiated glands. The present studies corroborate the view that the adrenal cortex is a radioresponsive tissue functionally, and indicate

that the effect of an ionizing radiation is exerted on all the steroidogenic enzyme systems of the biosynthetic mechanism.

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REFERENCES

BURSTEIN, S.: *Analyt. Chem.* **25**: 422. 1953.

DRAEGER, R. H., R. H. LEE, T. E. SHEA F. I. WHITTEN AND M. EICHER: Naval Medical Research Institute NM 006 012.04.64, July, 1953.

HECHTER, O.: *Endocrinology* **42**: 285. 1948.

HECHTER, O., R. P. JACOBSEN, R. JEANLOZ, H. LEVY, C. W. MARSHALL, G. PINCUS AND V. SCHENKER: Recent Progress in Hormone Research VI (Academic Press Inc., New York), p. 215, 1951.

HOLTORFF, A. F. AND F. C. KOCH: *J. Biol. Chem.* **135**: 377. 1940.

PORTER, C. C. AND R. H. SILBER: *J. Biol. Chem.* **185**: 201. 1950.

UNGAR, F., G. ROSENFELD, R. I. DORFMAN AND G. PINCUS: *Endocrinology* **56**: 30. 1955.

IRRADIATION AND ADRENAL STEROIDOGENESIS:[1]

INFLUENCE OF IRRADIATION OF ISOLATED ACTH-STIMULATED CALF ADRENALS ON THEIR CORTICAL OUTPUT

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INTRODUCTION

THE adrenal gland, particularly the cortex, has been implicated in many bodily responses to various physiological and pathological stimuli, including radiation. With respect to the latter agent, it is notable that the adrenal cortex, even when exposed to massive doses of radiation, does not manifest pronounced morphological changes as evidenced by standard histological staining techniques. This morphological standard of evaluation of the cortical response to radiation has given rise to the opinion that the adrenal cortex is a radioresistant tissue (Bloom, 1945). Histochemical studies, however, involving lipoid staining, have revealed some changes in the irradiated adrenal cortex suggestive of an altered adrenocortical function.

In view of the limitations of histological and histochemical techniques in revealing the actual dynamic state of adrenocortical activity in irradiated glands, the present investigation was undertaken as a direct biochemical approach to ascertain the effect of ionizing radiation on adrenocortical function by studying the corticosteroidogenic mechanism of irradiated adrenal glands. Pincus, Hechter and Zaffaroni (1950) have demonstrated that ACTH stimulates adrenal steroidogenesis when the isolated gland is perfused. We have employed this method to study the influence of gamma radiation from radiocobalt on the biogenesis and secretion of corticoids by irradiated, isolated calf adrenals perfused with homologous blood containing added ACTH. It was hoped that the experimental approach would pro-

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[2] Opinions and assertions are those of the authors and do not necessarily reflect those of the U. S. Naval Service.

vide specific information concerning the primary effect of ionizing radiation on the adrenocortical hormone-secretory mechanism and products without the complication of extrinsic factors which may be encountered *in vivo*, such as (a) an altered responsivity of the adrenocortical activating mechanism in the anterior pituitary and/or central nervous system, (b) a modified metabolism of the corticoids by non-adrenal tissues, or (c) secondary radiation effects from toxic products derived from extra-adrenal tissues.

MATERIALS AND METHODS

Perfusion of calf adrenal glands. Fresh calf blood and adrenal glands were obtained from a local slaughterhouse on the day of perfusion. A group of four glands consisting of two left and two right adrenals was perfused and simultaneously exposed to gamma radiation, while the two right and two left contralateral control glands were similarly perfused without irradiation. The cannulation and perfusion procedures were the same as those employed by Hechter (1948). Prior to adrenal passage, several liters of homologous citriated blood were perfused through isolated rat livers to remove vasoconstrictor and other undesirable substances. A liter of this prepared blood was then perfused through each group of four glands. Blood flow rates measured during the course of the perfusions were not significantly different for the irradiated and control glands. ACTH (Upjohn), equivalent in potency to one unit of Armour's LA-1A, was added hourly to the blood perfusion media. Following the perfusion, the adrenal effluents were immediately frozen in a dry ice-acetone mixture and kept in a frozen state until thawed and extracted for corticoids.

Irradiation. In each experiment, a group of four glands was perfused while being exposed to 4π gamma radiation from Co^{60} in the Radiocobalt Large Animal Irradiator of the Naval Medical Research Institute (Draeger *et al.*, 1953). The primary beam had an energy of 1.23 Mev. (with less than 10% softer scattered radiation). The dose rate ranged from 14.4 to 17 roentgens per minute in air as measured on a Victoreen rate meter with a 4 mm. lucite cap. The average dose rate to the organs was approximately in the same range.

Blood extraction. The blood perfusates were extracted with ethyl acetate (Meyer, 1953) as follows: the liter sample of frozen blood was thawed and maintained at 4° C. Following the addition of sodium chloride (150 g./l.), a 200 ml. portion of blood was extracted for 10 seconds with 600 ml. of ethyl acetate previously cooled to 4° C. The blood was removed, and a second 200-ml. portion added and extracted with the same 600 ml. of ethyl acetate, the process being repeated until the entire liter was extracted. The blood was then re-extracted in the same manner with a second sample of 600 ml. of ethyl acetate. A loss of 10–16% of the ethyl acetate into the aqueous phase occurred mainly during the first blood extraction.

The combined ethyl acetate extracts were washed serially with 50 ml. of water, 50 ml. of 0.1 N sodium hydroxide, and, finally, with three 50 ml. portions of water. The extract was then dried with sodium sulfate and evaporated to dryness under vacuum. The residue was dissolved in 70 ml. of methanol, which after the addition of 30 ml. of water was extracted three times with 30 ml. of petroleum ether in order to remove the lipids. The methanol was removed under vacuum and the remaining aqueous suspension extracted thoroughly with chloroform. The chloroform extract was dried with sodium sulfate and evaporated *in vacuo* to a residue. A light yellow oil was obtained which

weighed about 10–30% of the original residue before the petroleum ether partition. Aliquots of this final residue were used for various colorimetric tests as well as for paper chromatographic separation of the various steroid zones.

Paper chromatography and estimation of hydrocortisone and corticosterone. The blood extracts were fractionated by a paper chromatographic system using toluene-propylene glycol (Zaffaroni *et al.*, 1950). Quantitative estimations of hydrocortisone and corticosterone were made as follows: duplicate aliquots of the prepared extract were placed on paper strips, 3 mg. by weight to 1 cm. of paper, and developed in a toluene-propylene glycol system for 24 hours. During this period, the zone containing corticosterone migrated 15–30 cm., whereas the hydrocortizone zone remained at the origin. After the strips were allowed to dry, the steroid zones were located under ultraviolet light, and strips (1/20th of the total width) were cut on both sides along the entire length of the chromatogram. These strips were developed with the triphenyl tetrazolium reagent to demonstrate the hydrocortisone and corticosterone zones. The corresponding zones on the main strips containing these two corticoids were cut out and eluted with methanol overnight. Quantitative colorimetric analyses were conducted with aliquots of these methanol eluates.

Corticoid analyses. Various colorimetric analyses were conducted with aliquots of the prepared blood extracts or with aliquots of eluates from paper chromatographic zones. The original Porter and Silber (PS) method (1950) was modified by allowing the unheated reaction mixture to stand overnight at room temperature before reading at 410 mμ. Samples were run in duplicate with hydrocortisone as a standard. The phosphomolybdic acid procedure of Heard and Sobel as modified by Burstein (1953) was employed, and duplicate samples run with corticosterone and hydrocortisone as standards. Formaldehyde formation from steroids (FS), following periodic acid oxidation, was determined by the procedure of Hollander (1951). Ultraviolet absorption at 240 mμ was determined on eluates of the paper chromatographic zones containing corticosterone and hydrocortisone.

EXPERIMENTAL

The validity of the extraction procedures and colorimetric assay methods was established by the recovery experiments shown in Table 1. Two mg. of hydrocortisone were added to 500 ml. of whole blood (Exp. A), 2 mg. of hydrocortisone added after the ethyl

TABLE 1. RECOVERY OF HYDROCORTISONE (F) AND CORTISONE (E) ADDED TO BLOOD OR BLOOD EXTRACT DURING THE BLOOD FRACTIONATION PROCEDURE

Recovery Exp.	Conditions	Blood sample	Periodic acid oxidation (FS)		Porter-Silber chromogens (PS)		Ultra-violet absorption (240 mμ)	
			Found μg.	Recovery %	Found μg.	Recovery %	Found μg.	Recovery %
A	2 mg. hydrocortisone added to 1000 ml. whole blood.	1000 ml. + 2 mg. F	1800	75	2000	85	2200	99
		1000 ml.	344		286		202	
B	2 mg. hydrocortisone added to the ethyl acetate extract of 1 l. of blood before alkali wash.	1000 ml. + 2 mg. F	2200	93	2285	99	2382	109
		1000 ml.	344		286		202	
C	1.95 mg. cortisone added to the extract of 500 ml. of blood following alkali wash prior to petroleum ether partition.	500 ml. + 1.95 mg. E	2120	104	2500	99	2868	97
		500 ml.	100		555		969	

acetate extraction, but before the 0.1 N NaOH wash (Exp. B), and 2 mg. of cortisone were added before the petroleum ether-70% methanol partition (Exp. C). A quantitative recovery was obtained after the individual steps as well as following the entire procedure.

Recovery experiments to evaluate the elution of the chromatographic zones of hydrocortisone and corticosterone according to the toluene system of Zaffaroni and the Bush benzene-methanol-water system (1952) yielded equally satisfactory results on standard solutions. However, in practice, the resolution of blood extracts by the Bush system was found to be unsatisfactory because of the large amounts of material that were employed and desirable for accurate assay.

A representative adrenal perfusion experiment is given in Table 2. Two groups of four

TABLE 2. PERFUSION OF NORMAL CALF ADRENAL GLANDS

A group of 4 calf adrenals (I), perfused for 3 hours with fresh hourly changes of a liter of homologous blood containing 1 unit of added ACTH. The 4 contralateral glands (II) were simultaneously perfused without ACTH

Group	Porter-Silber chromogens secreted* μg./l./hr.		
	1st hr.	2nd hr.	3rd hr.
I. ACTH-stimulated glands	800	1350	750
II. Unstimulated control glands	200	250	650
Net output due to ACTH	600	1100	100

* Calculated by subtracting the blood chromogen values before perfusion from those found after perfusion.

glands were perfused with liter samples of calf blood, one without ACTH and the contralateral group with 1 unit of ACTH added per liter of blood per hour. The organs were perfused for three hours with fresh changes of blood each hour. The values represent net adrenocorticoid output and were calculated by subtracting the Porter-Silber chromogen content of the calf blood before adrenal perfusion from the content found after perfusion. The difference between the corticoid output of the ACTH-stimulated glands and the unstimulated contralateral controls represents the net production of corticoids due to ACTH stimulation. These values were substantiated by the relative intensities of the hydrocortisone and corticosterone zones developed after paper chromatographic separation of the extracts from perfusates of the stimulated and unstimulated glands.

RESULTS

Table 3 is a summary of the radiation doses and gland weights of six experiments in which groups of four calf adrenals were exposed to 4 π gamma radiation from Co^{60} while being perfused in a multicycle system with homologous blood containing ACTH. For each perfused group of irradiated glands, there was a group of unirradiated, ACTH-stimulated, contralateral controls simultaneously perfused under the same conditions. In Experiments 1 and 2, the glands were stimulated with ACTH throughout a two-hour perfusion period. In Experiments 3 through 6, the glands were irradiated and perfused without ACTH for a preliminary period of one hour.

TABLE 3. IRRADIATION DOSES AND ADRENAL WEIGHT DATA OF THE ACTH STIMULATION EXPERIMENTS

Exp. No.	Weight of 4 glands		Irradiation data		
	Irradiated gm.	Control gm.	Dose rate* r/min.	Exposure time min.	Total dose r
1	14.79	14.45	17	106	1802
2	13.73	13.86	17	111	1887
3	13.77	13.96	16.6	160	2656
4	14.61	14.39	16.6	165	2739
5	14.64	14.42	14.4	162	2333
6	11.73	11.91	14.4	163	2347

* As measured in air on a Victoreen rate meter.

After one hour, ACTH was added to the circulating blood, and the perfusion and irradiation continued for two more hours.

Table 4 summarizes the analytical values of the hydrocortisone and corticosterone as determined by the phosphomolybdic acid reaction following paper chromatography. A significantly lower secretion by the irradiated adrenals of both hydrocortisone and corticosterone was found in five experiments, in which their output was from 22 to 85% less than that of the unirradiated control glands. The Porter-Silber chromogenic values of the prepared blood extracts revealed similar differences in output. In five of the six experiments, the irradiated adrenals secreted from 22 to 96% less chromogen than the unexposed contralateral organs. An unexplained larger output of Porter-Silber chromogens was observed in one experiment. The Porter-Silber chromogen values of the unchromatographed blood extracts (Table 4) approximate the combined chromogenic values obtained

TABLE 4. INFLUENCE OF IRRADIATION OF ISOLATED ACTH-STIMULATED CALF ADRENALS ON THEIR CORTICOID OUTPUT

Exp. No.	Treatment of perfused calf adrenals	Porter-Silber chromogens in blood extract		Eluted paper chromatographic zones determined by phosphomolybdic acid					
				Hydrocortisone (F)		Corticosterone (B)		Compounds F and B	
		Amount μg.	Change* %	Amount μg.	Change* %	Amount μg.	Change %	Amount μg.	Change* %
1	Unirradiated 1802 r	260 390	+50	96 130	+35	141 128	− 9	237 258	+ 9
2	Unirradiated 1887 r	495 125	−74	411 97	−76	256 128	−50	767 225	−71
3	Unirradiated 2656 r	2171 856	−61	1565 268	−83	478 111	−77	2043 379	−81
4	Unirradiated 2739 r	624 379	−39	73 103	+41	402 219	−45	475 322	−32
5	Unirradiated 2333 r	1132 888	−22	1075 827	−23	1179 932	−21	2254 1759	−22
6	Unirradiated 2347 r	2375 89	−96	1461 100	−93	1094 281	−74	2555 381	−85

* Percentage decrease in output due to gamma irradiation from Co^{60}.

for corticosterone and hydrocortisone by the phosphomolybdic acid reaction on the paper eluates. Some of the discrepancies are due, in part, to non-specific chromogen material reacting with different intensities in the colorimetric methods.

The identity of the corticosterone zone was established by comparing mobility rates with that of a standard, run simultaneously. The hydrocortisone zone remained close to the starting line of the paper chromatograms and is known to contain other steroids beside hydrocortisone. The hydrocortisone zone was eluted in four experiments, and rechromatographed in a toluene-propylene glycol system for 96 hours. This procedure revealed three minor zones other than hydrocortisone. The purified hydrocortisone determined on this rerun represented 82% of the material in the first chromatogram.

DISCUSSION

The meager and controversial observations of the susceptibility of the adrenal cortex to radiation damage have been based mostly on indirect functional tests or on the static morphological criteria of histological and histochemical alterations. Although the cortex of adrenals irradiated *in vivo* does not usually exhibit marked anatomical changes, the present studies suggest that from a functional point of view, the adrenal cortex may well be considered a radio-responsive tissue. The data presented demonstrate that gamma irradiation of the isolated, ACTH-stimulated adrenal gland induces a significant decrease in its steroidogenic activity. Irradiated calf adrenals secreted an appreciably smaller quantity of hydrocortisone and corticosterone than the unexposed contralateral glands when both were perfused with homologous blood containing added ACTH. The radiation doses employed in these studies, if delivered to the whole body, would produce 100% mortality within four days in most mammals, but they cannot be compared with those used *in vivo* for obvious reasons. In view of the fact that *in vitro* studies have shown that doses of the order of 10^4–10^5 roentgens are generally required for pronounced effects, such as inhibition of growth or respiration in tissue cultures, the doses herein employed must be considered as relatively moderate.

The reason for the reduction in steroidogenesis induced by irradiation is not apparent at this time. Studies are now in progress to elucidate whether the diminished secretion of such metabolically important corticoids as hydrocortisone and corticosterone is related to a defect in the synthesis of the steroid nucleus, or in the activity of the 11β-, 17α-, and 21-hydroxylases, or to a combination of these possible defects. The experimental approach involves the perfusion of model steroids, i.e., DOC, compound S, 21-dexoxy E, progesterone, and dehydroepiandrosterone, to as-

certain any differences in the specific biosynthetic enzymatic reactions of irradiated and normal adrenals by isolating and determining the major transformation products derived from the perfused steroid substrates.

SUMMARY

Exposure of the isolated, ACTH-stimulated calf adrenal to moderate doses of gamma radiation (Co^{60}) during perfusion resulted in a significantly decreased secretion of hydrocortisone, corticosterone, and unidentified Porter-Silber chromogens. Although the cortex of irradiated animals does not exhibit marked morphological alterations, the present studies suggest that from a functional point of view, the adrenal cortex may be considered a radioresponsive tissue.

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REFERENCES

BLOOM, W.: Histopathology of Irradiation from External and Internal Sources (McGraw-Hill Book Co., Inc.). 1948.

BURSTEIN, S.: *Analyt. Chem.* **25**: 422. 1953.

BUSH, I. E.: *Biochem. J.* **50**: 370. 1952.

DRAEGER, R. H., R. H. LEE, T. E. SHEA, F. I. WHITTEN AND M. EICHER: Naval Medical Research Institute NM 006 012.04.64, July, 1953.

HECHTER, O.: *Endocrinology* **42**: 285. 1948.

HOLLANDER, V. P., S. DIMAURO AND O. H. PEARSON: *Endocrinology* **49**: 617. 1951.

MEYER, A. S.: *J. Biol. Chem.* **203**: 469. 1953.

PINCUS, G., O. HECHTER AND A. ZAFFARONI: Proc. II Clinical ACTH Conf. Vol. I. Ed. J. R. Mote. (The Blakiston Co., New York), p. 40. 1950.

PORTER, C. C., AND R. H. SILBER: *J. Biol. Chem.* **185**: 201. 1950.

ZAFFARONI, A., R. B. BURTON AND E. H. KEUTMANN: *Science* **111**: 6. 1950.

THE METABOLISM OF THYROTROPHIC HORMONE IN THE RAT[1]

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MANY researches have clearly established the important regulatory influence of the thyroid stimulating hormone (TSH) of the adenohypophysis on the growth and secretion of the thyroid gland. The primary actions of the hormone on virtually all phases of thyroid function have been intensively investigated, and relatively few species have escaped experimental scrutiny in this regard (Adams, 1946; Albert, 1949; D'Angelo, 1954). The precise mode of action of TSH on the thyroid cell is nevertheless unknown. There is much uncertainty also regarding the locus of degradation and metabolic fate of this trophic principle in the organism. Although this area of investigation has received limited attention, "in vitro" experiments have yielded results which are generally considered to suggest that the thyroid itself plays an important role in the metabolism of thyrotrophin. Seidlin (1940) found that TSH was removed from solutions in contact with suspensions of beef thyroid tissue. Rawson, Sterne and Aub (1942) observed that incubation of rabbit thyroid tissue with thyrotrophin resulted in practically complete inactivation of the hormone. A variety of other tissues tested were ineffective except thymus and lymph node which partially inactivated the hormone. In similar studies on normal and pathologic human thyroids, TSH inactivation by explants of normal thyroid tissue was again demonstrated (Rawson, Graham, Riddell, 1943). Thyroid tissue from patients with Graves' disease had greater inactivation properties than normal whereas non-toxic nodular goiters (colloid type) failed to inactivate TSH. Rawson and Money (1949) more recently have shown that thyrotrophin inactivated by exposure to rabbit thyroid tissue can be reactivated by reducing agents. These authors have tentatively hypothesized that in producting its effect on the thyroid at the cellular level thyrotrophic hormone becomes inactivated in an unknown reaction, presumed to be oxidative in nature.

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The advent of bioassay methods which can detect endogenous as well as exogenous TSH in the circulating blood (D'Angelo and Gordon, 1950) has permitted a direct approach to the problem of the "in vivo" metabolism of the hormone. D'Angelo (1951) determined the disappearance rate of exogenous thyrotrophin from the blood of normal and hypophysectomized rats. The ephemeral nature of the hormone in the circulation of the normal rat contrasted markedly with its persistence in the blood of the hypophysectomized animal. The relatively slow removal of TSH from the latter was attributed to a retardation in rate of hormone destruction consequent to a general metabolic change in the organism rather than to specific alteration in the thyroid gland. The present study attempts to test this hypothesis by determining whether or not the hyperplastic thyroid of rats treated with antithyroid agents can alter disappearance rates of administered TSH from the circulation, and whether thyroid hormone or other calorigenic agents can appreciably influence blood TSH recoveries in the hypophysectomized animal.

MATERIALS AND METHODS

Adult female and male albino rats of the Wistar strain were used exclusively in the experiments with propylthiouracil (PTU). The goitrogen was fed in the purina ration (.05%) for periods of 2, 3, 4, and 10 months to induce varying degrees of thyroid hyperplasia. Hypophysectomized rats (males, Sprague Dawley) were used 4–6 months postoperatively. Oxygen consumption (BMR) was measured by the closed circuit method of Grad (1952). Determinations were made at least 6 times to establish a base level after which the calorigenic agents were given. Groups of hypophysectomized rats received a single subcutaneous injection of either l-thyroxine[2] (TH: 2 mg. per kilo. body weight) or l-triiodothyronine[2] (TIT: 2 mg. per kilo. body weight). The 2,4-dinitrophenol (DNP) was given intraperitoneally (20 mg. per kilo. body weight). In other experiments with pituitaryless rats, TIT was administered daily (100 μg.) for 8 days. Intravenous administration of TSH[3] was made after BMRs were elevated to supernormal levels. In thyroid hormone treated rats, thyrotrophin was usually given within 24–48 hours after the last injection. Because of the transient effect of dinitrophenol on oxygen consumption TSH was administered within 1–2 hours after injection of this agent.

TSH was given to goitrous rats and intact controls in one injection (inferior vena cava) after nembutal anesthesia. Hypophysectomized rats were injected under ether anesthesia via the jugular vein. Rats received either 1 mg. of TSH in acidified saline (1 cc.) or saline alone. In some experiments, PTU and TIT were given in a single intravenous injection immediately following administration of thyrotrophin. One half hour following TSH injection, blood was withdrawn from the heart without anticoagulant, the bloods of rats in each group pooled, and the sera obtained frozen until ready for bioassay. At autopsy, the thyroids were quickly removed and trimmed, weighed, and prepared for histologic study.

[2] Obtained from Smith, Kline and French Laboratories, courtesy of Dr. M. Sampson, Philadelphia, Pa.

[3] Generously supplied by Dr. W. Donaldson, Parke, Davis and Company, Detroit, Mich.

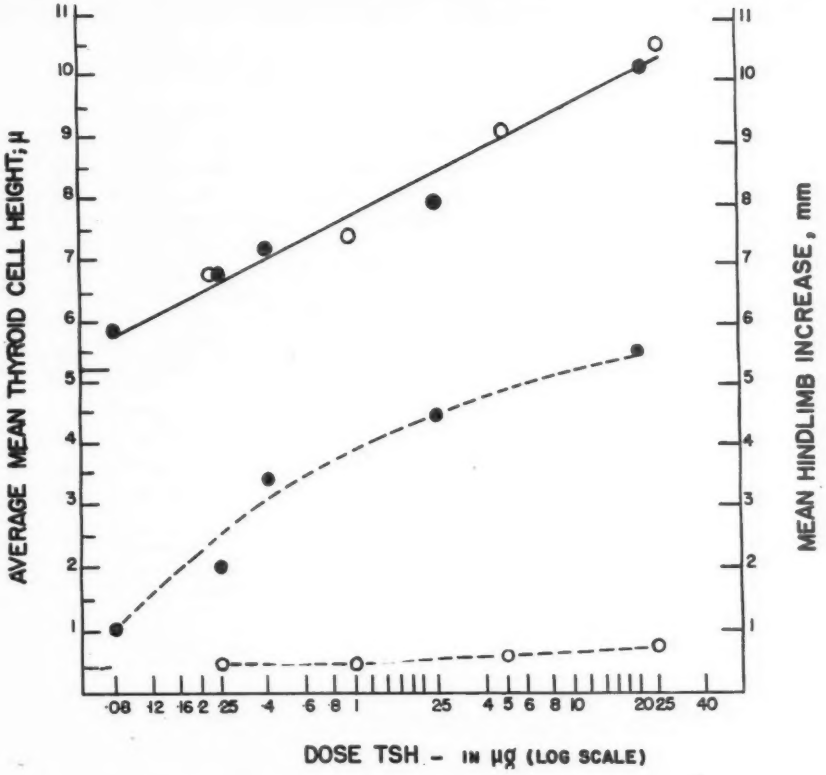

FIG. 1. Log dose-response curve of the standard TSH preparation (3–5 Junkmann-Schoeller units per milligram). The increase in thyroid cell height of the stasis tadpole is shown by the solid line. (Open circles represent assays on Rana clamitans and closed circles those on Rana pipiens; slopes of the curves and their variance for both species will be analyzed elsewhere. A single regression line fitted to both sets of points introduces only negligible error in the present experiments.) Hindlimb response (broken line) is appreciably different in both species and is not used as a secondary criterion of TSH detection here. Horizontal bars on the ordinate represent values in the control tadpole.

Bioassay of the standard TSH preparation and sera was done by modification of the stasis tadpole technic.[4] Rana clamitans as well as Rana pipiens tadpoles were used, with intraperitoneal injections made on successive rather than alternate days. The dose response curve was obtained by serial dilution of the hormone in acidified saline, the total dose of TSH given over the 4 day-injection period ranging from 0.1 to 25 μg. The response of the thyroid was measured microhistometrically and recorded as acinar cell height increase (effects on hindlimb extension are disregarded with this abbreviated

[4] Technical assistance from Miss Rose Nakayama and Mrs. Ada Schafer is acknowledged gratefully.

method). The sera of hormone or saline treated rats were similarly bioassayed (.05 ml. daily, 4 days).

Per cent recovery of exogenous TSH in sera was estimated according to the equation:

$$\frac{\text{TSH concentration detected—endogenous TSH concentration}}{\text{Theoretically expected TSH concentration}} \times 100 = \text{percent recovery}$$

The absolute amount of TSH in the sample was found by extrapolation of the mean thyroid cell height value, together with its standard error, on the standard nomogram (Fig. 1). From this, the mean TSH concentration in serum (with upper and lower limits detected) could be calculated. Inasmuch as this represented both exogenous and any endogenous TSH present in the sample, values for the latter (arrived at by similar extrapolation by cell heights in saline injected controls) were subtracted. Exogenous TSH concentration was then expressed as a per cent of the theoretically expected concentration, this being calculated in turn on the assumption that the 1000 μg. of administered hormone are distributed in a plasma volume equivalent to 2.4% of the body weight.

RESULTS

A significant rise in blood titers of endogenous TSH occurred in all rats chronically treated with PTU (Table 1). Some indication of a reduction

TABLE 1. RECOVERY OF EXOGENOUS TSH IN THE BLOOD OF THE GOITROUS RAT

Group	Number rats (sex)	Mean body weight (gm.)	Mean thyroid wgt. (mg.) / 100 gm. body	Bioassay of TSH: Mean thyroid cell hgt.	Bioassay of TSH: Mean conc. TSH (μg./ml. serum)	Mean % recovery TSH
Normal+Sal.	6 (♀)	201	7.4	5.8±0.12[1]	0.3 (.25–.4)[2]	—
Normal+TSH	3 (♀)	223	6.8	8.3±0.22	9.5 (7–13)	4.9
2 Months PTU+TSH	3 (♀)	227	30.1	9.5±0.10	40 (35–45)	21.7
2 Months PTU+Sal.	3 (♀)	214	25.3	6.4±0.03	0.8 (0.7–0.9)	—
Normal+Sal.	4 (♀)	196	7.0	5.6±0.14	<0.3	
Normal+TSH	3 (♀)	226	4.7	8.5±0.25	13 (9–17.5)	7.1
3 Months PTU+TSH	3 (♀)	224	35.1	9.5±0.24	40 (30–55)	20.6
3 Months PTU+Sal.	3 (♀)	193	26.2	6.5±0.18	0.9 (0.7–1.5)	—
Normal+Sal.	3 (♂)	275	4.9	5.7±0.06	<0.3	—
Normal+TSH	3 (♂)	246	6.5	8.4±0.35	12 (7–17.5)	6.9
4 Months PTU+TSH	3 (♂)	270	43.6	9.5±0.40	40 (25–80)	25.2
4 Months PTU+Sal.	3 (♂)	260	46.9	6.5±0.13	0.9 (0.8–1.2)	—
4 Months PTU—Off 3 days+TSH	4 (♀)	205	32.6	9.5±0.28	40 (30–55)	19.2
Normal+TSH	5 (♀)	194	7.3	8.1±0.19	7 (6–9)	3.1
10 Months PTU+TSH	3 (♂)	332	48.4	9.4±0.36	35 (22.5–55)	27.4
10 Months PTU+Sal.	3 (♂)	365	44.6	6.1±0.12	0.5 (0.4–0.6)	—
Normal+Sal.	3 (♂)	335	5.8	5.9±0.10	0.35 (0.3–0.5)	—
Normal 10 mg. PTU +TSH[3]	4 (♀)	241	5.2	7.3±0.24	4 (2.7–5.3)	2.1
Normal TSH+Sal.	4 (♀)	218	6.0	6.9±0.28	2.5 (1.7–3.5)	1.3

[1] Average-mean acinar cell height in micra ± standard error of mean, based on 7–9 test tadpoles in each bioassay.
[2] Mean TSH concentration in terms of standard preparation; in parentheses, values for range of detection as calculated from the standard error of each assay.
[3] PTU and TSH given simultaneously in a single intravenous injection.

toward normal levels appeared after 10 months of goitrogen administration however. Despite the progressive hyperplasia of the thyroid, the elevation in blood TSH levels was of the same order of magnitude at the various time intervals studied.

The amount of exogenous TSH which could be recovered in the sera of

goitrous rats one half hour following its intravenous administration was consistently and substantially higher than normal (Table 1). Mean recoveries of the hormone from the blood of normal animals, measured in 5 separate experiments, ranged from 1–7% of theoretically expected values as calculated from the standard dose response curve (Fig. 1). In comparable experiments with rats bearing thyroids 4–8 times heavier than normal, TSH recoveries averaged 19–27%. The amounts of exogenous TSH recoverable bore no apparent relationship to thyroid size. Thyroid hyperplasia at 4 and 10 months of PTU treatment was approximately twice greater than that after 2–3 months of goitrogen administration but mean recoveries of TSH were 26 and 21% respectively. The amount of administered hormone which could be detected in the blood of goitrous animals from which PTU had been withdrawn (3 days previously) approximated that for rats which received the goitrogen continuously. In contrast, the conjoint administration of a single dose of PTU and TSH into the blood stream of normal rats yielded a hormone recovery value in the normal range.

Mean TSH recoveries from the blood of hypophysectomized rats treated with calorigenic agents are shown in Table 2. It is evident that the amount

TABLE 2. EFFECT OF CALORIGENIC AGENTS ON THE RECOVERY OF EXOGENOUS TSH FROM THE BLOOD OF HYPOPHYSECTOMIZED RATS*

Treatment	Mean body[2] wgt. (gm.)	Mean thyroid wgt. (Mg.)	Mean BMR (cc. O_2/hr./100 gm.)		Bioassay TSH	
			Initial[3]	Final[4]	Mean conc. TSH (μg./Ml. serum)[5]	Mean % recovery TSH
TSH	144	7.3		116	125 (70–138)	43.2
TIT[1]+TSH	145	6.5		114	138 (120–150)	48.8
TIT+TSH	128	7.1	118	167	35 (30–45)	10.8
DNP+TSH	177	8.2	108	154	48 (30–75)	20.4
TH+TSH	168	8.3	97	145	15 (10–23)	6.0
TIT+TSH	160	7.7	108	164	5.5 (4–6)	2.1

* 4–6 months post-operative.
[1] Single intravenous injection immediately following TSH.
[2] 3–4 rats per group.
[3] Prior to treatment.
[4] One-half hour before TSH administration.
[5] Mean TSH concentration in terms of standard preparation; in parentheses, values for range of detection as calculated from the standard error of each assay.

of hormone which can be recovered in the sera at the ½ hour interval after injection is significantly less in rats in which oxygen consumption was appreciably increased by previous treatment with calorigenic substances. Thyroxine and triiodothyronine were more effective than dinitrophenol in decreasing the recovery of TSH despite the fact that the BMR was raised to equally high levels from the pre-operative value. In contrast, the simultaneous administration of TSH and TIT in a single intravenous injection

yielded a blood TSH recovery which approximated that with TSH alone. The acceleratory effect of thyroid hormone on the rate of disappearance of exogenous TSH from the circulation of the hypophysectomized rat evidently requires previous elevation of the oxidative metabolism to normal or super-normal levels. Superimposition of the $\frac{1}{2}$ hour recovery values obtained in the present study on previously determined curves for disappear-

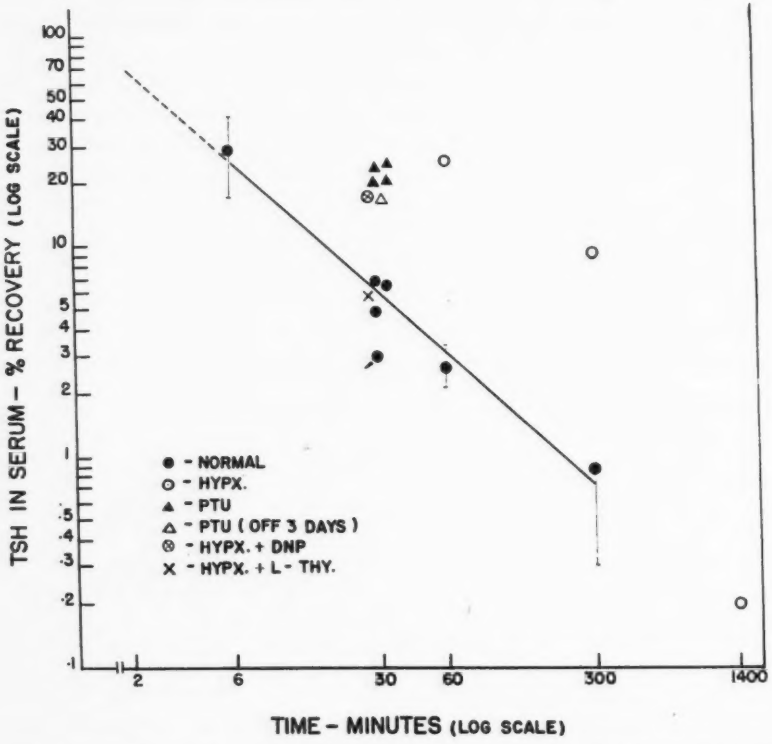

FIG. 2. The recovery of exogenous thyrotrophin from the blood is shown for normal, goitrous, and hypophysectomized rats. The curve describes the rate of disappearance of the administered hormone in the normal animal. TSH recovery values for time intervals other than 30 minutes are based on previous data (D'Angelo, 1951).

ance rates of TSH in normal and hypophysectomized rats shows reasonably good correspondence (Fig. 2). However, the TSH content of blood in the untreated hypophysectomized rats, as well as in those given TIT simultaneously with the exogenous TSH, was so high at the $\frac{1}{2}$ hour interval that thyroid cell height increase in the test tadpole lay at the extreme upper limit of the dose response curve. Slight revision upward of TSH

recovery values in these animals would probably render them more accurate. By the same token, the relatively low TSH recoveries in rats with elevated metabolism become still more significant.

DISCUSSION

The finding that exogenous TSH persists in the circulation of goitrous rats is difficult to reconcile with the hypothesis that the thyroid gland is the primary locus of degradation of thyrotrophic hormone. Although extrathyroidal sites of action of TSH have been occasionally suggested, there is no question that the thyroid gland is the primary target for TSH. Evidence for any specific inactivation of TSH at the thyroid level is largely inferential however, and is based primarily on the "in vitro" experiments cited above. Although it is likely that the thyroid does inactivate some thyrotrophic hormone under "in vivo" conditions, a primary role in this regard is highly questionable. Sonenberg, Keston, Money and Rawson (1952) found that localization of radioactivity after the intravenous administration of an S^{35} labeled TSH preparation to the young chick occurred in a variety of tissues. Concentrations of radioactivity in thyroid and gonad were relatively high but only a small fraction of that found in the liver. Richards and Sayers (1951) were unable to implicate the adrenal to any appreciable degree in the rapid disappearance of ACTH (exogenous) from blood. The total ACTH recovered in the kidney was considerably greater than for the adrenal although concentrations in the latter were high.

The results obtained in goitrous and hypophysectomized rats strongly suggest that the role of the thyroid in the phenomenon of TSH inactivation can be at best only a subsidiary one. In both experimental conditions, circulating levels of exogenous TSH remain high in the presence of either an atrophic or hyperplastic thyroid. There is no reason to believe that the enlarged thyroids of PTU-treated animals are insensitive to exogenous TSH so as to spare destruction of the hormone. The goiters themselves are the results of TSH (endogenous) stimulation; moreover, it is known that the conjoint administration of a goitrogen with TSH may augment thyroid response. PTU enhances the effect of exogenous TSH on the iodide-trapping function of the thyroid (Halmi, Spirtos, Bogdanove, Lipner, 1953) and potentiates the TSH-induced depletion of iodine from the gland (Vanderlaan and Greer, 1950). The persistence of exogenous TSH in the circulation of the goitrous rat thus can hardly be explained on the basis of lack of interaction with its target organ. Indeed, it is likely that the slow rate of disappearance of TSH in the goitrogen treated animal may account, partially at least, for the effects just cited. In view of the chemical reactivation of TSH which can be effected with goitrogenic agents (Albert and associates, 1947a, 1947b), the question arises as to whether the high

recoveries of TSH in the PTU-treated rat result from a direct chemical interaction between hormone and goitrogen "in vivo." The present study appears to answer this in the negative. Recovery of TSH was much above normal in rats from which PTU had been withdrawn for 3 days prior to hormone administration. The simultaneous injection of TSH and PTU into the circulation, moreover, did not alter appreciably recovery values of the hormone in the normal animal.

The slow rate of disappearance of thyrotrophin from the blood of the hypophysectomized or goitrous rat, like that shown for the thyroidectomized rabbit (Loeser, 1934), may well be a reflection of some fundamental metabolic change common to all three experimental conditions. In each instance, the animal is deficient in thyroid hormone and exists at a low metabolic level. It is significant that in the hypophysectomized rat, with atrophic thyroid, disappearance rate of TSH from the blood could be markedly accelerated by pre-treatment with thyroxine or triiodothyronine (the relative effectiveness of these substances was not tested). No tissues have as yet been specifically implicated but it is conceivable that the sparing of blood TSH in the thyroid hormone deficient animal results from retardation of some inactivation process in the tissues. This may reflect a specific type of metabolic alteration rather than the general lowering of the oxidative metabolism. That the former may be significant is indicated by the fact that dinitrophenol was much less effective than thyroid hormone in hastening the removal of exogenous TSH from the circulation. Other calorigenic agents need be tested, however, before the *character* of the metabolism can be considered of primary importance in the disappearance of thyrotrophin from the blood.[5] The effectiveness of thyroid hormone in promoting the removal of exogenous TSH from the circulation provides a tentative explanation for the well known phenomenon that the simultaneous administration of thyroid and thyrotrophic hormones to the intact animal (Loeb, Bassett, and Friedman, 1930; Aron, VanCaulert, and Stahl, 1931) or to the hypophysectomized one (Cortell and Rawson, 1944) induces milder histologic change in the thyroid gland than with thyrotrophin alone. The more rapid disappearance of TSH from the blood would result in effectively lower concentrations stimulating the target gland. Under these circumstances, the morphologic response of the thyroid should be less vigorous.

[5] It should be pointed out that other factors may influence the concentration of hormone in the plasma at a fixed interval of time following injection. Distribution and equilibration rates of thyrotrophic hormone in the various bodily compartments—about which virtually nothing is known at present—are possibly affected in the goitrous or hypophysectomized rat. Other hemodynamic alterations, such as change in blood volume, would also result in differences in TSH plasma concentration not necessarily reflecting metabolism or degradation of the hormone.

SUMMARY AND CONCLUSIONS

Quantitative estimation of endogenous and exogenous thyrotrophic hormone in the blood of normal, goitrous, and hypophysectomized rats has been made by means of the stasis tadpole technic of bioassay.

Endogenous TSH blood titers were found to be significantly elevated in rats chronically treated with propylthiouracil. The rise did not parallel the thyroid hyperplasia.

Mean per cent recoveries of a single dose of intravenously administered TSH from the circulation of goitrous rats were consistently greater than normal.

The slow rate of disappearance of exogenous TSH from the blood of hypophysectomized rats could be accelerated by previous elevation of the metabolism with calorigenic agents. Thyroxine and triiodothyronine were much more effective than dinitrophenol in hastening the removal of thyrotrophic hormone from the circulation.

The persistence of exogenous TSH in the blood of rats with either hyperplastic or atrophic thyroids does not support the hypothesis that the thyroid gland plays a major role in the inactivation of circulating hormone. The concept is offered that the removal of thyrotrophin from the blood is primarily influenced by metabolic levels in tissues responsive to the thyroid hormone.

REFERENCES

ADAMS, E. A.: *Quarterly Review of Biology* **21**: 1. 1946.

ALBERT, A. *Ann. N. Y. Acad. Sci.* **50**: 466. 1949.

ALBERT, A., R. W. RAWSON, P. MERRILL, B. LENNON AND C. RIDDELL: *Endocrinology*, **40**: 299. 1947a; 303. 1947b.

ARON, M., C. VANCAULERT AND J. STAHL: *Compt. rend. Soc. Biol. Paris.* **107**: 64. 1931.

CORTELL, R. AND R. W. RAWSON: *Endocrinology* **35**: 488. 1944.

D'ANGELO, S. A.: *Endocrinology* **48**: 249. 1951.

D'ANGELO, S. A.: Brookhaven Symposia in Biology, in press.

D'ANGELO, S. A. AND A. S. GORDON: *Endocrinology* **46**: 39. 1950.

HALMI, N. S., B. N. SPIRTOS, E. M. BOGDANOVE AND H. J. LIPNER: *Endocrinology*, **52**: 19. 1953.

LOEB, L., R. B. BASSETT AND H. FRIEDMAN: *Proc. Soc. Exper. Biol. and Med.* **28**: 209. 1930.

LOESER, A.: *Arch. Exp. Path. u. Pharmakol.* **176**: 697. 1934.

RAWSON, R. W., G. O. STERNE AND J. C. AUB: *Endocrinology* **30**: 240. 1942.

RAWSON, R. W., R. M. GRAHAM AND C. B. RIDDELL: *Ann. Int. Med.* **19**: 405. 1943.

RAWSON, R. W. AND W. L. MONEY: Proceedings of the Laurentian Hormone Conference **4**: 397. 1949.

RICHARDS, J. B. AND G. SAYERS: *Proc. Soc. Exper. Biol. and Med.* **77**: 87. 1951.

SEIDLIN, S. M.: *Endocrinology*, **26**: 696. 1940.

SONENBERG, M., A. S. KESTON, W. L. MONEY AND R. W. RAWSON: *Jour. Clinical Endocrinology and Metabolism* **12**: 1269. 1952.

VANDERLAAN, W. P. AND A. M. GREER: *Endocrinology* **47**: 36. 1950.

ELECTRON MICROSCOPIC OBSERVATIONS ON THE THYROID GLANDS OF NORMAL, HYPOPHYSECTOMIZED, COLD-EXPOSED AND THIOURACIL-TREATED RATS[1]

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RECENT advances in fixation (Palade, 1951) embedding (Newman, Borysko and Swerdlow, 1949) and sectioning (Pease and Baker, 1948; Latta and Hartman, 1950; Dempsey and Lansing, 1953) have made it possible to prepare thin sections of almost any mammalian tissue for use in the electron microscope. With the current techniques and instruments, resolutions of 100 Å are easily possible, so that approximately twenty times the definition available with the ordinary microscope can now be attained. We have initiated, in this laboratory, a program of electron microscopy of various tissues and organs the physiological activity of which can be controlled by experimental means, with the intent of correlating changes in function with whatever alteration occurs in the morphological elements of cells. The present investigation concerns the submicroscopic structure of the rat's thyroid gland. In the following passages we shall describe the appearance of the thyroid follicular cells in states of inactivity and in stages during which thyroid colloid is being formed rapidly. This study, we believe, throws light upon the mechanisms and morphological elements involved in the secretory process.

MATERIALS AND METHODS

Twelve normal and three hypophysectomized young adult male rats weighing approximately 120 gm. were secured from the Endocrine Laboratories, Chicago, Illinois. The hypophysectomized animals were killed seven days after operation, the completeness of which was verified by examination of the sella turcica at autopsy. Three of the normal rats were killed as controls. Three were maintained in wire cages in a cold-room kept at $5° \pm 1°$ C. for seven days and then killed. Two rats were kept at normal room temperature but were given 0.1% propylthiouracil (Lederle), in their drinking water for 3 days, two for 6 days, two for 9 days and the remaining two for 12 days.

The rats were killed with an overdose of ether. The thyroid glands were rapidly

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dissected out and fixed in Palade's fluid (1% osmic acid buffered with veronal to pH 7.5). After fixation for 4 hours, the tissues were dehydrated through ethanol, embedded in a mixture of one part methyl- to three parts butyl-methacrylate which was polymerized at 45° C. under the influence of benzoyl peroxide. Thin sections were cut with glass knives on the microtome described by Dempsey and Lansing (1953). The sections were floated onto a water drop, lifted on collodion-coated copper-mesh grids, and examined in the electron microscope without removing the plastic. The electron microscope was an RCA model EMU2c which had been modified by introducing a centerable condenser aperture and an objective aperture, the diameter of which was .001 inch. The specimens were photographed at initial magnifications of 1000 to 5000 diameters. Subsequently, photographic enlargements were made as desired.

RESULTS

The Normal Follicle. Our observations are in general accord with the previous accounts published by Monroe (1953) and Braunsteiner, Fellinger and Pakesch (1953). However, examination of the illustrations in these publications indicates that the sections utilized were too thick for the best resolution and that the methods of embedding and subsequent removal of the embedding material caused a significant amount of distortion. Our material, embedded in methacrylate and examined directly without removal of the plastic, introduces less distortion and permits recognition of many details obscured in the photographs cited above.

The free, lumenal border of the thyroid cell exhibits a somewhat dome-shaped appearance and is bounded by a dense plasma membrane. This membrane is frequently thrown into minute, fingerlike elevations or microvilli. These microvilli are circular in cross-section, and contain an outer, dense pellicle continuous with and indistinguishable from the plasma membrane. The centers of the villi have a density comparable to that of the apical cytoplasm, and they are continuous with it (Figs. 1, 3 and 5).

Immediately beneath the apical border, a narrow zone characterized by rather homogenous small granules of moderate density is usually encountered. Mitochondria, colloid vacuoles and ergastoplasm ordinarily are absent from this zone, although occasionally one of these larger complexes invade the region and occur immediately under the plasm membrane. This zone sometimes appears swollen. When it does, it contributes to the dome-shaped contour of the apical border of the follicular cell.

Mitochondria, recognizable by their moderate electron density, their circular or ovoid outlines and by their dense and characteristic internal structure (Palade, 1952), are encountered throughout the cytoplasm of follicular cells. They display no particular orientation or concentration in any cellular region, but rather seem to be randomly located.

Frequently, but not in all cells, spherical electron-dense bodies are found in the supra-nuclear cytoplasm. Their size is about the same as that of mitochondria. Their size, distribution and osmiophilia suggest their iden-

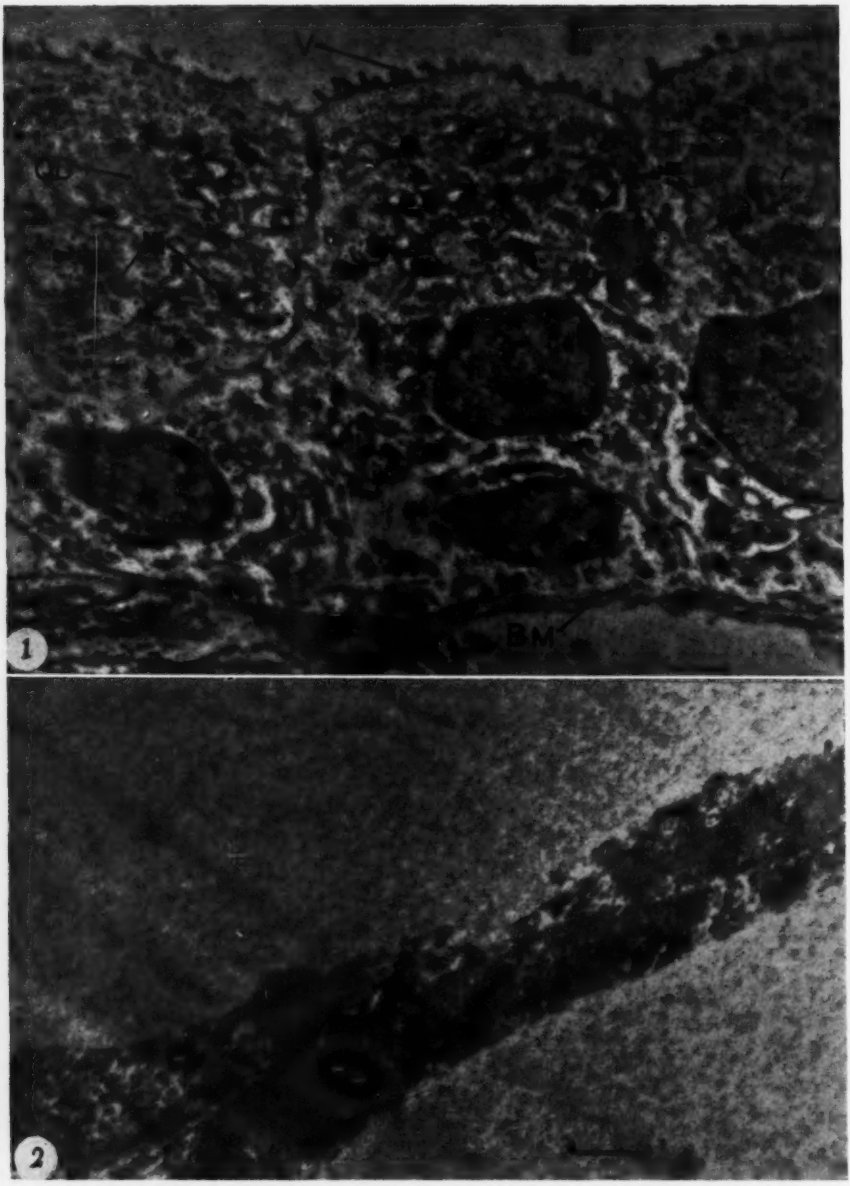


FIG. 1. Slightly tangential cut through a follicle from a normal rat. The colloid is at the top of the figure. Microvilli (V) project from the apical surface of the cells into the colloid. Beneath the microvilli and the surface membrane, a relatively clear zone containing small granules can be seen. Colloid droplets (CD), mitochondria (M) and ergastoplasmic sacs or vesicles (E) are labelled. At the base of the cell the thin basement membrane (BM) is visible. The clear space at the lower right corner is a plasma-filled capillary, lined with a very thin layer of endothelium. ×10,000.

FIG. 2. Section through the thyroid from an animal hypophysectomized 7 days previously. The thinning of the follicular cells, the density of the cytoplasm and colloid and the reduction of cell organelles are evident. ×12,000.

FIG. 3. Thyroid follicular cell from a rat kept in a cold-room at 5° C. for 7 days. Microvilli (V), the superficial clear granular zone, the Golgi region (G) and the colloid droplets (CD) are evident. The colloid droplets are more numerous than in the normal thyroid, their density approximates that of the colloid, and they are surrounded by dark membranes similar to those of the ergastoplasm. ×12,000.

tity with the lipid droplets frequently encountered in thyroid cells (Dempsey, 1949).

The most striking cytoplasmic component is a system of complex structures bounded by long and tortuous dark lines. These structures vary greatly in their disposition, appearing sometimes as a pair of lines which course together for long distances maintaining rather accurately a parallel course. In other instances, these twin lines diverge from one another into sac-like structures which may or may not exhibit diverticulae. These sacs sometimes appear empty; sometimes they contain a homogeneous gray material which matches the density of the colloid and at times even surpass it. The sacs may be minute or quite large. The largest ones, which approach a micron in diameter, almost invariably are filled with a gray homogeneous colloid material. Their appearance is such as to leave little doubt that they correspond to the colloid droplets of light microscopy.

Close examination of the twin lines bounding the smaller and more flattened of these sac-like structures reveals that the lines are associated, on their outer margins, with small, very dense granules. These granules are customarily aligned with some regularity on the membrane of the sac. Similar, small, dense granules are interspersed in the cytoplasms matrix between the sacs.

These observations leave little doubt that the system described here is similar to that encountered in many other cells. Bernhard, Hagenau, Oberling and Gauthier (1952) and Dalton, Kahler, Striebach and Lloyd (1950) have described such elements, which they have called "lamellar" or "canalicular," in pancreatic, hepatic and gastric cells. Porter (1953) has observed similar elements in many tissues and has named them the "endoplasmic reticulum." All of the above authors agree that the structures occupy the basophilic portions of cells and suggest that they represent, therefore, the structural configuration of the cytoplasmic ribonucleoprotein. Weiss (1953) called attention to the historical term "ergastoplasm" as descriptive of the basophilic portions of cells and referred to these formations as ergastoplasmic sacs and to the system of sacs and their associated granules as the ergastoplasmic complex. The latter author also showed that the complexes were destroyed by ribonuclease, a fact which further supports the hypothesis that they represent the cytoplasmic nucleoproteins. Monroe (1953) and Braunsteiner *et al.* (1953) both have observed the ergastoplasmic structures in the thyroid gland and both accept, at least tentatively, the hypothesis that they represent the cytoplasmic ribonucleoprotein and that they are somehow concerned with protein synthesis.

In addition to the small dense granules associated with the ergastoplasmic complexes, the cytoplasm contains numerous other granular elements. These are dispersed in a rather random fashion throughout the cell. The

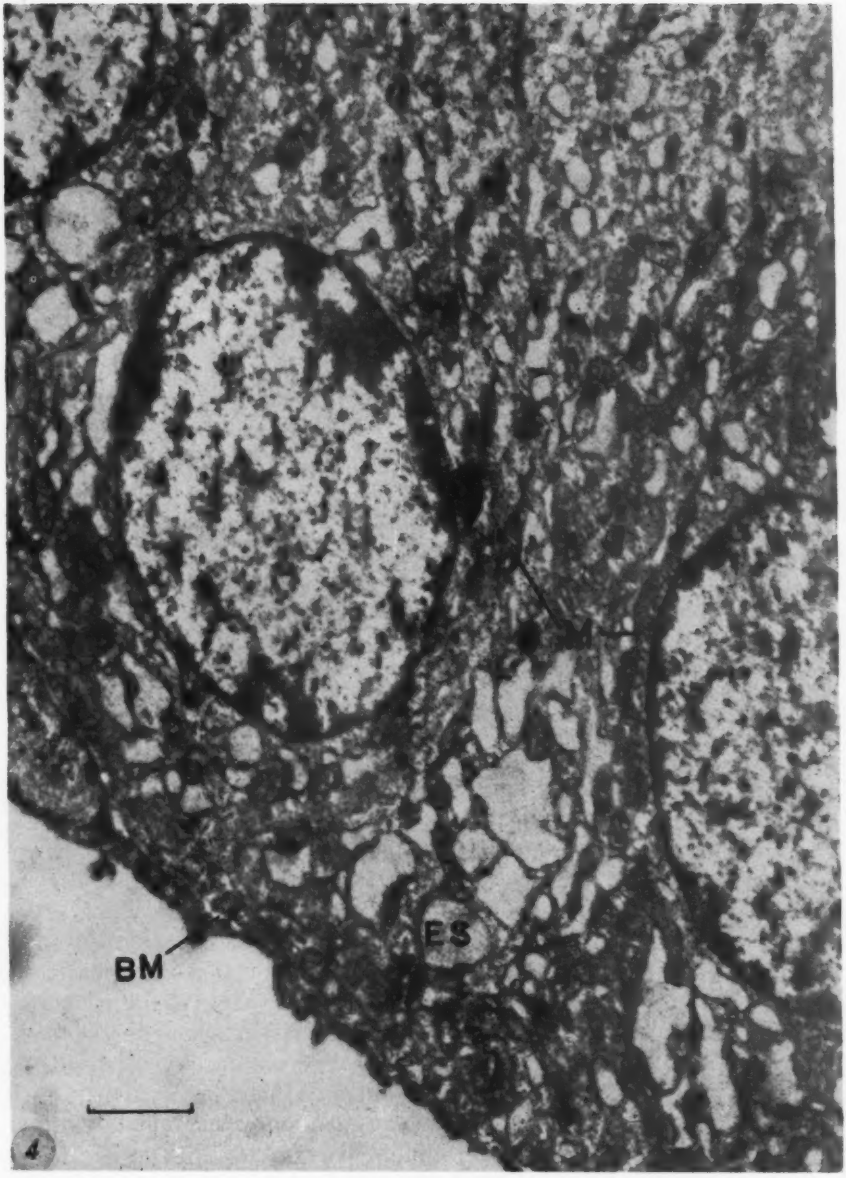


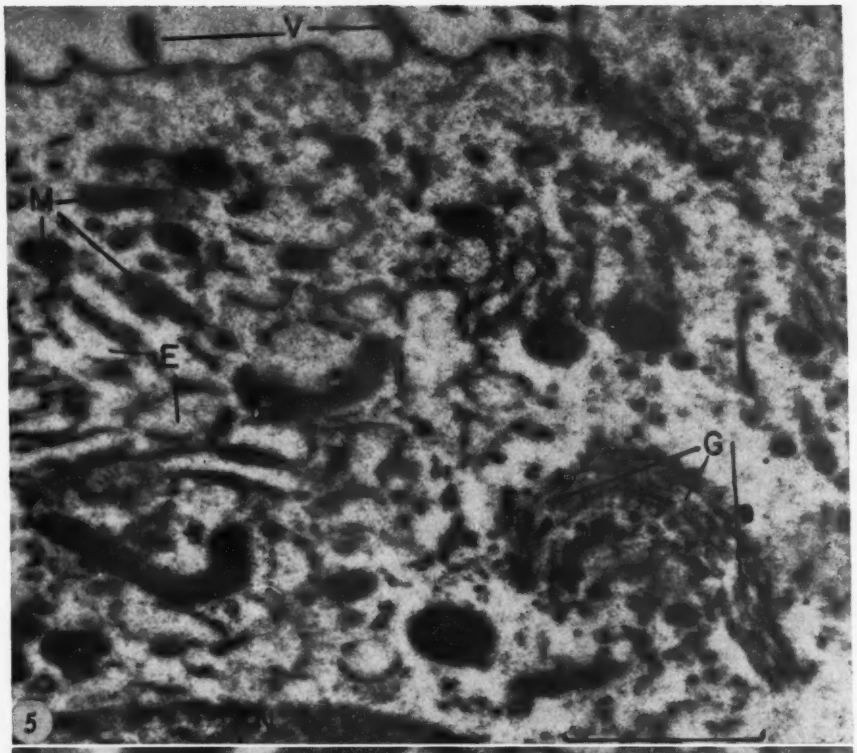
FIG. 4. Basal portion of a follicular cell from a rat given propylthiouracil for 9 days. The increase in vesiculation of the ergastoplasm (ES) gives the cell a lacy appearance. Mitochondria (M) are also abundant. The basement membrane (BM) can be distinguished as a thin line just above the flattened endothelium, a nucleus of which appears along the left-hand margin of the figure. ×15,000.

granules are less dense and less well defined than are those associated with the ergastoplasmic sacs. They represent, presumably, the precipitated amorphous substances of the ground cytoplasm.

Here and there in our micrographs there occurred an ill-defined but characteristic structure which most probably represents some portion of the Golgi complex. Figure 5 illustrates the appearance of this structure. It is composed of non-granular membraneous elements, sometimes aligned with respect to one another to give a lamellar appearance, sometimes appearing as tiny vesicles either in solitary occurrence or amalgamated together into a foamy structure, and sometimes with its membraneous elements dispersed loosely rather than packed together as shown in the illustration. These structures are most frequently encountered in the supranuclear portion of the cell, and, less frequently, along the lateral margins of the nuclei. They have never been seen in the infranuclear pole nor in the most apical portion of the cytoplasm. They occur, therefore, in the Golgi zone. We have not been able to identify them in the thyroids from hypophysectomized rats. In normal animals, these structures are seen only occasionally and then are small, whereas they appear to the best advantage in the glands from animals maintained in the cold or treated with thiouracil. The Golgi apparatus, being larger in states of activity, parallels these observations. Finally, these structures are similar in many respects to those studied by Dalton and Felix (1954) and identified by them as belonging to the Golgi complex.

The nuclei of the normal follicular cells are spherical in shape. They are filled with ill-defined chromatin masses of granular material. These granules are of varying shapes, sizes and densities; however most of them are quite dense and compare favorably in that regard with the granules of the ergastoplasmic complexes. The nuclear membrane presents a hair-line appearance, and in favorable areas can be resolved into a double line, each of which is approximately 50 Å thick, separated by a clear space about 100 Å wide. Bennett and Porter (1953) describe similar double-lined nuclear walls in the nuclei of striated muscle fibers. They mention that the appearance could be caused by visualizing the upper and lower edges of a single membrane cut in a slightly oblique plane. This explanation we are unable to accept, since it fails to account for the relatively constant dimensions of the twin-lined structure, and since other single membranes cut obliquely, such as the plasma membrane, do not present such an appearance but rather appear merely to broaden in their oblique portions.

The basement membrane of the thyroid gland has been the subject of dispute among microscopists for many years. Most textbooks state that a true basement membrane does not exist but that the base of the follicular cell abuts directly upon the capillary endothelium, the interfollicular con-



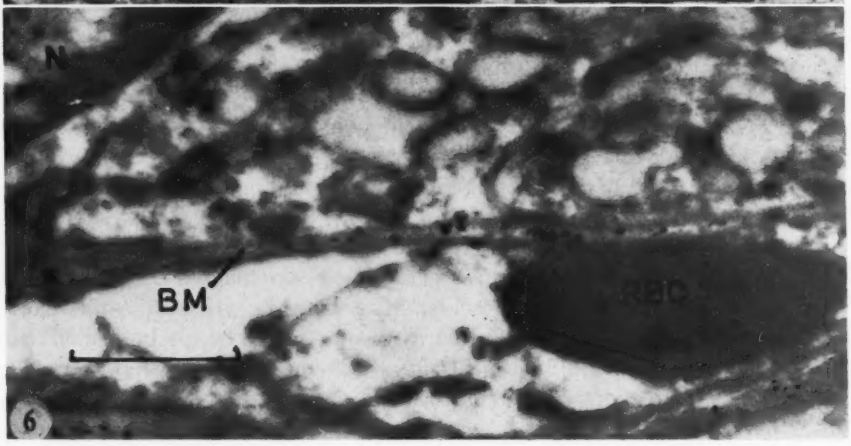


FIG. 5. Apical portion of a follicular cell from a rat exposed to cold for 7 days. Microvilli (V) projecting into the intrafollicular colloid may be seen at the top of the figure. Mitochondria (M) and ergastoplasm (E) are labelled. At the bottom of the figure, a small part of the nucleus (N) is shown. In the supranuclear region a condensation of tiny, membraneous structures occupies the Golgi region (G). ×30,000.

FIG. 6. Portion of the base of a follicular cell from a rat fed 0.001% $AgNO_3$ in its drinking water for 10 months. The silver has deposited as metallic granules in the basement membrane (BM) separating the endothelium from the base of the follicular cell. An erythrocyte (RBC) is located in the capillary lumen. Part of the nucleus of the follicular cell (N) is included for orientation. ×25,000.

nective tissue or, in situations where two follicles are closely approximated, upon the base of the subjacent follicular cell. Our micrographs, however, exhibit a gray, amorphous band of material closely applied to the base of the follicular cell but distinct from the basal plasma membrane (Figs. 1, 3 and 6). A similar basement membrane, approximately 200 Å in thickness, surrounds the capillary. Between the two membranes a space of variable thickness occurs in which occasional wisps of collagenous fibrils occur. This space is continuous with the interfollicular connective tissue in which fibroblasts, mast cells and other connective tissue elements are located.

Other, and physiological, evidence also exists for the existence of a basement membrane in the thyroid gland. Studies on the deposition of silver in clinical and experimental argyria have revealed that the metal is located in basement membranes in many parts of the body (Hill and Pillsbury, 1939; Wislocki and Leduc, 1952). More recently, we have examined with the electron microscope tissues from rats experimentally fed dilute solutions of silver nitrate for many months (Dempsey and Wislocki, in preparation). In the thyroid from these animals, silver deposits occur in thin, homogenous zones at the base of the thyroid follicular cell and surrounding the capillary endothelial cells (Fig. 6). This experimental situation therefore reveals a thin but active barrier between the blood vessel and the follicular cell, and demonstrates that in the living state it possesses a functional capacity.

The capillary meshwork surrounding the thyroid follicle is quite rich, forming a wreath of anastomosing vessels closely applied to the follicular epithelium. Both in the light microscope and in electronmicrographs, these capillary vessels can be seen frequently to indent the base of the follicular cell, so that the individual capillary may be nearly surrounded by epithelium. Such an intraepithelial capillary supply, although unusual, is by no means unique to the thyroid gland. Similar arrangements are found in the vaginal and cervical mucous membranes as well as occasionally in skin.

The endothelium lining the capillaries varies greatly in thickness from place to place. At the nodal points where nuclei are located the endothelium attains a considerable thickness. Mitochondria, ergastoplasmic sacs and a variety of cytoplasmic granular precipitates can be distinguished. In other locations, the endothelium becomes extremely thin, so that at times its thickness is no greater than that of its underlying basement membrane. Interspersed between the thin regions are nodular thickened loci, with occasional small mitochondria and other cytoplasmic structures enclosed within them. Monroe (1953) has suggested that, at places, the endothelium is actually discontinuous. This we regard as unlikely, since close examination of the thinnest areas in our micrographs exhibit always a continuity of the endothelium except where palpable breaks have occurred upon excessive stretching of the section or upon the expansion caused by

excessive exposure to the electron beam. Since the resolution in Monroe's micrographs is approximately the same as the dimensions of the thinnest areas, and since in her specimens the embedding material was removed by sublimation, that is, by exposure to an intense electron beam, we are inclined to attribute the discontinuities illustrated by her to low resolving power or to artifacts of preparation, or both.

The Thyroid After Hypophysectomy. The flattening of the follicular cells and the consequent compression of the nuclei, well known from ordinary microscopy, are noteworthy in our micrographs. The substance of the follicular cells becomes remarkably dense so that even our thinnest sections appear quite dark, with their elements closely packed together. The cells look as if they had lost much of their fluid matrix with the result that their solids have become much more concentrated (Fig. 2). The ergastoplasmic system has become reduced in amount and the sacs, where present, are collapsed together so that their lumens are nearly obliterated. The small dense granules of the ergastoplasmic complex also are reduced in number, leaving the cytoplasm packed with the more indiscriminate granular matetrial of the ground cytoplasm. Microvilli, although occasionally present on the apical borders of the follicular cells, are less noteworthy than in the cells from normal rats. No especial change has been noted in the capillary endothelium, although the capillaries do not indent the follicular cells but rather are merely apposed to the peripheral surfaces of the follicles.

The Thyroid After Exposure To Cold. The most noteworthy change occurring after cold stimulation is the increased height of the follicular cells.

Within the cells, the ergastoplasmic sacs are dilated so that many parts of the cell assume a lace-work appearance. Between these dilated lumens the membraneous ergastoplasmic sacs and their associated granules are found, the sacs being apposed to one another so closely that they frequently appear superficially to be a double-membraned system. Close examination, however, reveals that the associated small granules lie between the membranes, and that the dilated lumens are completely surrounded by the membrane. Thus, the close approximation is fortuitous, being merely the results of one sac approaching another because of the swelling of the structure.

The lumens of the sacs are not empty, but contain a homogenous, colloid-like substance of slight to moderate electron density (Fig. 3). This substance is similar in appearance to the colloid, and is interpreted as representing the colloid droplets which are known to increase in number during stimulation by cold.

The Thyroid After Stimulation By Propylthiouracil. The glands from animals to which thiouracil had been administered exhibited the same changes as those described for the cold-exposed rats. However, the altera-

tions were more extreme (Fig. 4). The ergastoplasmic sacs were greatly increased in number and in dilatation so that entire cells, except for their nuclei, assumed the characteristic lacy appearance. The contents of the sacs, and the colloid in the follicular lumens, was less dense than in the normal glands. The mitochondria were enlarged, especially as compared with those from hypophysectomized animals. The homogenous matrix material was less dense in these enlarged mitochondria, with the result that the internal sulci or plicae were more readily visible.

DISCUSSION

The observations reported in the previous sections confirm and extend much of the current knowledge concerning thyroid cytology. The height and density of cells, the number and size of mitochondria and the Golgi complex, the presence of colloid vacuoles and the alteration in these elements in different functional states were known from studies with the light microscope and are now found to be easily confirmable with the electron microscope. Similarly, the occurrence of microvilli on the free surface of the cells, the extreme thinness of the capillary walls, the presence of organized, structural elements corresponding to the basophilic cytoplasmic ribonucleoproteins, all have been mentioned previously in the studies of Monroe (1953) or of Braunsteiner *et al.* (1953) and have been readily confirmed by us in the present study. We differ slightly from Monroe (1953) in her interpretation of the thin walls of the capillaries; she speculated that a discontinuous endothelium formed an incomplete lining of the vessel, whereas we have not obtained appearances such as those illustrated by her except in situations in which gross mechanical distortion of the tissue had occurred. We therefore regard that the thyroid gland has a closed circulation, albeit the endothelial lining may be of extraordinary thinness.

The question of a basement membrane upon which the follicular cells rest would seem to have been resolved by the electron microscope studies. All three of the accounts mention a thin homogenous membrane at the base of the cells. Monroe's observations would seem further to provide evidence for its mechanical toughness; if our interpretation is correct that the discontinuities in the endothelium are caused by mechanical distortion, then the lack of similar distortion in the basement membrane would indicate that it has an inherent toughness greater than that of the cellular elements. Our observation that silver is deposited in the basement membrane of animals fed silver nitrate raises some interesting questions as to its physiological properties. In histochemical preparations, basement membranes in general are argyrophilic and possess reducing activity. Although few direct analytical data are available, what there are suggest that basement membranes belong to the family of mucopolysaccharides found

throughout connective tissues. Since the silver, administered in ionic form, is observed in metallic form, the basement membrane traps it in granular aggregates after its reduction in the living animal. The basement membrane apparently acts as a layer of reducing activity, or as a filter, or as both.

Perhaps the most interesting of our observations pertain to the membraneous character of the ergastoplasmic and the Golgi complexes, and to the fact that the colloid droplets are surrounded by a membrane. The larger colloid droplets contain material the density and appearance of which is identical with the intrafollicular colloid. If we accept that the smaller and less dense droplets represent the forming colloid, that is, the secretion precursors, it would follow that the thyroid secretion is formed and segregated within a membrane-covered vacuole. Moreover, the membraneous walls of these vacuoles appear to be derived from the ergastoplasmic sacs in that complete transition stages can be detected. In other words, the ergastoplasmic sacs, with their granular membranes, first become dilated, the contents of the sacs become denser and the sacs round up into spherical droplets. The membranes surrounding the droplets become less granular as their contents becomes more dense until the final stage is reached in which the fully formed droplet is enclosed in a smooth membrane. The fully formed droplets occur in all parts of the thyroid cell, and consequently our observations throw no light on the problem of apical *vs* basal secretion.

Several observations point to the ergastoplasmic, rather than the Golgi elements, as the origin of the droplet membranes. The Golgi membranes are invariably smooth and non-granular, whereas the droplets of slight density have granular membranes. The Golgi membranes are associated with tiny vesicles whereas the ergastoplasmic vacuoles are of a size roughly corresponding to that of the droplets. The Golgi material is restricted to the supranuclear region of the cell, wheras droplets in all stages of density are encountered in all regions, including the base, where ergastoplasmic sacs are numerous.

The association of secretory products and ergastoplasmic elements described here for the thyroid is similar in most respects to the situation depicted by Weiss (1953) for the pancreatic zymogen granules. In his study, small vesicles were observed to bud off from the larger ergastoplasmic sacs; these became filled with a colloid mass which gradually enlarged and became denser until it became transormed into the zymogen granule. In the present study, the ergastoplasmic sac itself was observed to fill with a colloid material. The processes are therefore parallel in all essential points. In both glands, the secretory products appear in the center of ergastoplasmic elements. Assuming an identity between the ergasto-

plasm and the cytoplasmic ribonucleoproteins, these observations provide morphological data on the association between nucleoproteins and protein secretion.

SUMMARY

Electron microscopy of thin sections from osmic-fixed, methacrylate-embedded thyroid glands has revealed numerous details concerning the structure of plasma membranes, mitochondria, granules, the Golgi region, the ergastoplasm and basement membranes. Many of these structures change in size, number or appearance in altered physiological states such as inactivity induced by hypophysectomy or hyperactivity resulting from treatment with thiouracil or exposure to cold. Colloid droplets have been observed; they possess an outer membrane derived from ergastoplasmic sacs. A basement membrane, thin but exhibiting a characteristic appearance and activity, is present at the base of follicular cells.

REFERENCES

BENNETT, H. S. AND K. R. PORTER: *Am. J. Anat.* **93**: 61. 1953.

BERNHARD, W., F. HAGENAU, A. GAUTHIER AND C. OBERLING: *Zeit. f. Zellforsch.* **37**: 281. 1952.

BRAUNSTEINER, H., K. FELLINGER AND F. PAKESCH: *Endocrinology* **53**: 123. 1953.

DALTON, A. J. AND M. D. FELIX: *Am. J. Anat.* **94**: 171. 1954.

DALTON, A. J., H. KAHLER, M. J. STRIEBACH AND B. LLOYD: *J. Nat. Cancer Inst.* **11**: 439. 1950.

DEMPSEY, E. W.: *Ann. N. Y. Acad. Sci.* **50**: 336. 1949.

DEMPSEY, E. W. AND A. I. LANSING: *Proc. Soc. Exper. Biol. & Med.* **82**: 253. 1953.

HILL, W. R. AND D. M. PILLSBURY: *Argyia. The Pharmacology of Silver.* Williams and Wilkins, Baltimore, 172 pp. 1939.

LATTA, H. AND J. F. HARTMAN: *Proc. Soc. Exper. Biol. & Med.* **74**: 436. 1950.

MONROE, B. G.: *Anat. Rec.* **116**: 345. 1953.

NEWMAN, S. B., E. BORYSKO AND M. SWERDLOW: *Science* **110**: 66. 1949.

PALADE, G. E.: *J. Exper. Med.* **95**: 285. 1951.

PALADE, G. E.: *Anat. Rec.* **114**: 427. 1952.

PEASE, D. C. AND R. F. BAKER: *Proc. Soc. Exper. Biol. & Med.* **67**: 470. 1948.

PORTER, K. R.: *J. Exper. Med.* **97**: 727. 1953.

WEISS, J. M.: *J. Exper. Med.* **98**: 607. 1953.

WISLOCKI, G. B. AND E. H. LEDUC: *J. Comp. Neurol.* **96**: 371. 1952.

EFFECT OF PYRIDOXINE DEFICIENCY ON THE GONADOTROPHIC CONTENT OF THE ANTERIOR PITUITARY IN THE RAT[1]

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NELSON, Lyons and Evans (1951) have previously shown that the reproductive disturbances observed in pyridoxine (B_6) deficiency are due to a lack of the ovarian hormones, estrin and progestin. This was demonstrated by the maintenance of pregnancy in B_6-deficient rats injected with the synthetic hormones, estrone and progesterone, at the dosage levels known to be effective in rats hypophysectomized and oophorectomized after breeding (Lyons, 1943). Attempts to maintain pregancy in B_6-deficient rats with the pituitary gonadotrophins were only partially successful, indicating that the function of both the pituitary and the ovary was affected by B_6-depletion (Nelson, Lyons and Evans, 1953).

To obtain information on the anterior pituitary, the glands of both B_6-deficient and normal rats have been bioassayed for the three gonadotrophic hormones. Ovarian function, as shown by the response to injected gonadotrophins will be considered in a later report.

EXPERIMENTAL METHODS

The anterior pituitaries of normal and of B_6-deficient female rats were dissected, weighed, ground, and injected as a suspension in isotonic saline with 2% butanol.[2] The pituitaries of 8 to 12 rats were pooled for each bioassay in order to minimize individual

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[2] The pituitary suspensions, with butanol added as a preservative, were stored for an average of two weeks in a refrigerator before using. Preparations may be stored for 60 days without detectable loss in gonadotrophic activity as judged by bioassay.

variations in hormone content which might be due to stage of the estrous cycle in normal rats or to degree of deficiency in B_6-deficient rats. The average weight of the anterior pituitaries from B_6-deficient rats was 7.9 mg. and that for normal anterior pituitaries was 10.9 mg. The glands were assayed for follicle stimulating (FSH) activity and for interstitial cell stimulating (ICSH) activity in hypophysectomized rats at dosage levels varying from $\frac{1}{4}$ to 6 glands and for mammotrophic (MH) activity in squabs at levels varying from 1/64 to one gland. Doses were given as fractions or multiples of anterior pituitaries rather than on a weight basis to facilitate a gland to gland comparison of the animals. Three to 5 test animals were used for each dosage level and most dosages were repeated at least once.

Immature female rats, hypophysectomized at 26–28 days of age and 11–23 days postoperative, were used as test animals to determine FSH and ICSH activity. The minimal effective dose (MED) for follicle stimulation and for interstitial cell stimulation was determined in a 96-hour test with 4 daily subcutaneous injections. The 96-hour instead of the standard 72-hour test period (Evans *et al.*, 1939) was used as the pituitary content of growth and of thyrotrophic hormones was being determined at the same time (to be reported in a later publication). The histological responses for FSH and ICSH as described by Evans *et al.* (1939) were used as the criteria.[3] The MED for follicle stimulation was that amount causing resumption of follicular development in 2 of 3 rats, *i.e.* increase in size of follicles with beginning antrum formation. The MED for interstitial cell stimulation was that amount which caused partial repair of "deficient" interstitial tissue in 2 of 3 rats, *i.e.* the resumption of the normal nuclear pattern in these cells with some increase in cytoplasm.

The anterior pituitary suspensions were bioassayed for mammotrophic activity in one-month old squabs by the sensitive "micro" method of Lyons and Page (1935). Whenever possible, tests of equivalent amounts from glands of normal and B_6-deficient rats were made in the same bird by injection over paired crop sacs. Crop sac spreads were stained and examined and the MED for crop stimulation was determined, *i.e.* a minimal response in 3 of 5 birds. In addition, the crops were graded on a scale ranging from $\frac{1}{2}+$ (minimum response) to 4+ (maximum response) so that activity at all dose levels could be compared.

The B_6-deficient rats used as pituitary donors were virgin females of the Long-Evans strain placed on the B_6-deficient diet at 80–90 days of age for two months or until approximately 150 days of age. Vaginal smears were examined daily in order to determine the regularity of the estrous cycle. The animals were weighed at regular intervals and were examined for acrodynia. Only those were selected which showed definite signs of B_6-deficiency, namely, cessation of growth, progressive acrodynia and loss of the estrous cycle. The normal rats used as donors averaged 150 days of age and had normal estrous cycles. Rats at all stages of the estrous cycle were used. Uterine and ovarian weights were determined for representative groups of pituitary donors and a comparative histological study was made of the ovaries from normal and B_6-deficient rats.

[3] The gonadotrophic activity of the pituitary is expressed in terms of the MED necessary to stimulate the hypophysectomized rat ovary rather than equivalents of units of purified FSH, ICSH, or MH. The rat unit for FSH and for ICSH, as defined by Evans *et al.* (1939) is based on a 72 hour test with subcutaneous injection of FSH and intraperitoneal injection of ICSH. The question of end points for a differential gonadotrophic assay in which the test for FSH is carried out in the presence, and even an excess, of ICSH has been discussed by Bahn *et al.* (1953). The bioassay procedure used in this study is similar to that used by Bahn and coworkers.

The B_6-deficient diet was the same as that used in previous studies (Nelson, Lyons and Evans, 1951, 1953). It consisted of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco or Primex), 4% salts,[4] and 0.5 mg.% desoxypyridoxine (a vitamin B_6-antagonist). Crystalline vitamins per kilogram of diet were: 300 μg. d-biotin, 5 mg. 2-methyl-1,4-naphthoquinone, 5 mg. thiamine HCl, 5.5 mg. pteroylglutamic acid, 10 mg. riboflavin, 10 mg. p-aminobenzoic acid, 20 mg. niacin, 50 mg. d-calcium pantothenate, 400 mg. inositol, and 1.0 gm. choline chloride. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units vitamin D, 6 mg. synthetic dl-alpha-tocopherol, and 650 mg. corn oil (Mazola). Normal animals received a stock diet of natural foodstuffs.[5]

RESULTS

The B_6-deficient rats selected as pituitary donors showed only a mild degree of inanition, averaging a loss of body weight of 10% or 19 gm. after 60 days of deficiency (Fig. 1). At the time of autopsy an average of 35 days

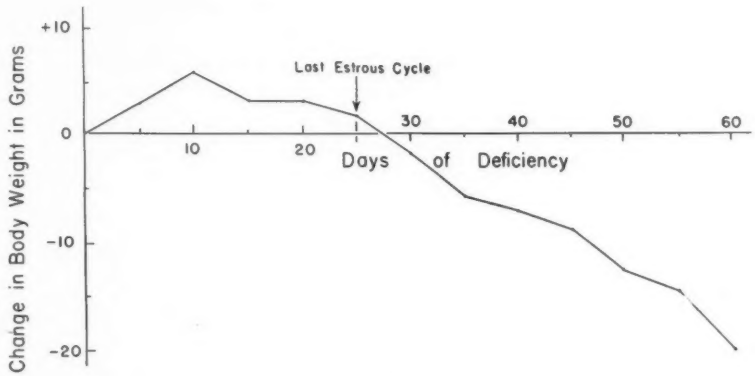

FIG. 1

had elapsed since the last estrus, as judged by vaginal cornification. The average uterine weight in the B_6-deficient group was 113 mg. (range 62 to 214 mg.); in the normal rats the average was 428 mg. (range 269 to 645 mg.). The uteri of B_6-deficient rats were pale and atrophic in appearance; the normal uteri showed the typical changes in vascularity and size associated with respective stages of the estrous cycle. The average ovarian weight for B_6-deficient animals was 33 mg. (range 17–58 mg.) and that of the normal animals 58 mg. (range 40–72 mg.).

Histologically, the ovaries of B_6-deficient rats were characterized by the

[4] Salts No. 4 of Hegsted *et al.* (1941)

[5] The stock diet, 14, is composed of 68.5% ground whole wheat, 10% fish meal, 10% alfalfa leaf meal, 4% casein, 5% fish oil, and 1.5% iodized NaCl. Lettuce is given *ad libitum* twice weekly.

presence of both healthy and atretic follicles, atrophic interstitial tissue, and persisting corpora lutea. The follicles, medium to medium-large in size, were comparable to those seen in the ovaries of normal adult female rats in diestrum, although fewer in number. In no case, however, did the follicles attain a size comparable to those observed in the pre-ovulatory stage of the normal ovary. The interstitial cells had pale-staining cytoplasm and pyknotic nuclei. Some "deficient" interstitial cells, or "wheel" cells with narrow cytoplasm similar to those seen in the hypophysectomized rat, were observed in every ovary. Corpora lutea in various stages of degeneration were present, the most recent showing more maintenance than would be expected considering the time which had elapsed since the last estrus. A similar persistence of corpora lutea has also been observed after hypophysectomy (Evans *et al.*, 1941).

The results of bioassays for FSH and ICSH activity in the anterior pituitaries of normal and of B_6-deficient rats are summarized in Table 1.

TABLE 1. FOLLICLE STIMULATING AND INTERSTITIAL CELL STIMULATING ACTIVITY IN THE ANTERIOR PITUITARIES OF NORMAL AND OF B_6-DEFICIENT FEMALE RATS

Pituitary dosage		Test animals[1]	Uterus wt.[2]	Ovaries[3]			Activity[4]	
No.	Wt.			Wt.	Follicles	Interstitial tissue	FSH	ICSH
	mg.	no.	mg.	mg.				
Normal Females								
0	0	33	15	7	s	deficient	Neg	Neg
¼	2.5	16	17	8	s	deficient	Neg	Neg
½	5.4	21	16	8	s	deficient	Neg	Neg
1	10.8	21	18	9	s	partial repair	Neg	MED
2	23.0	9	18	10	s	repair	Neg	>MED
4	40.4	7	17	9	s	repair	Neg	>MED
6	65.6	8	33	19	m, few l, some CL	repair	>MED	>MED
B_6-Deficient Females								
0	0	23	15	7	s	deficient	Neg	Neg
¼	1.7	8	14	7	s	deficient	Neg	Neg
½	3.7	19	17	9	s	partial repair	Neg	<MED
1	6.7	21	25	15	sm, some m-l	repair	>MED	>MED
2	17.0	6	76	43	m, l, some CL	repair	>MED	>MED
3	25.5	3	93	51	m, l, some CL	repair	>MED	>MED

[1] Immature females, hypophysectomized 26–28 days, 11–23 days postoperative.
[2] The oviduct was removed and the uterus drained before weighing.
[3] Abbreviations used are as follows: CL = corpora lutea; MED = minimal effective dose; s, m, l refer to the size, small, medium or large, of follicles.
[4] See text for explanation of MED for FSH and ICSH activity.

ICSH activity could be detected in normal glands at the one pituitary dosage level (10.8 mg.). This dosage resulted in partial repair of the interstitial tissue in practically all test animals, a response which we have considered as the MED for interstitial cell stimulation. Increasing the dose to 2 and 4 glands caused complete repair of the interstitial tissue. FSH was detected in normal glands at the 6 pituitary dosage level (65.6 mg.). At this dosage some medium and a few large follicles were produced in most of the test animals, a degree of follicular development in excess of that considered as

the MED. Luteinization of follicle walls and formation of corpora lutea with enclosed ova occurred in some ovaries. The average ovarian weight increased from 7 mg. in uninjected animals to 19 mg. in injected rats. At this dosage level (6 pituitaries) the average uterine weight increased also, from 15 to 33 mg., indicating estrin production. In view of these findings, 5 glands were considered to be the MED of normal anterior pituitary tissue necessary for follicle stimulation, and one gland as the MED for interstitial tissue repair (Table 3).

ICSH was first detected in B_6-deficient glands at the one-half pituitary level (3.7 mg.) (Table 1). Partial repair of the interstitial tissue occurred in less than half of the test animals at this level, a response not sufficient to be considered as the MED for interstitial cell stimulation. When the dose was increased to one pituitary, the interstitial tissue was completely repaired in all animals, a response in excess of that representing an MED. FSH activity was detected in B_6-deficient glands at the one pituitary level (6.7 mg.). Two-thirds of the ovaries showed at least minimal follicular stimulation, and in one-third medium to large follicles were observed, the response representing more than an MED for follicle stimulation. The average ovarian weight increased from 7 to 15 mg. at this dosage level. Estrin production also occurred at the one pituitary level, the average uterine weights increasing from 15 to 25 mg. in injected rats. When the dose was increased to 2 or 3 pituitaries, further increases in follicular development and in ovarian and uterine weight occurred. At these higher dosage levels luteinization of follicle walls and formation of corpora lutea with enclosed ova occurred in all ovaries. The MED of B_6-deficient anterior pituitary tissue necessary for follicle stimulation and for interstitial cell stimulation was, therefore, considered to be $\frac{3}{4}$ of a gland (Table 3).

TABLE 2. MAMMOTROPHIC ACTIVITY IN THE ANTERIOR PITUITARIES OF NORMAL AND OF B_6-DEFICIENT FEMALE RATS

Pituitary dosage	Normal			B_6-deficient		
	Pituitary dosage	Crops[1] tested	Crop sac response[2]	Pituitary dosage	Crops tested	Crop sac response
no.	mg.	no.		mg.	no.	
$\frac{1}{64}$	0.1	3	0–$\frac{1}{2}$+	0.1	3	0
$\frac{1}{32}$	0.3	8	$\frac{1}{2}$–1+	0.2	13	$\frac{1}{2}$+
$\frac{1}{16}$	0.7	5	1$\frac{1}{2}$+	0.5	10	1+
$\frac{1}{8}$	1.4	3	1+	1.1	5	1$\frac{1}{2}$+
$\frac{1}{4}$	2.8	5	2+	2.1	5	3+
$\frac{1}{2}$	7.2	5	2$\frac{1}{2}$–3+	4.4	5	2$\frac{1}{2}$+
1	13.7	5	2$\frac{1}{2}$–3+	9.9	5	2$\frac{1}{2}$+

[1] The crop sacs of one-month old Silver or White King squabs, weighing approximately one pound.

[2] Ratings of $\frac{1}{2}$+ (minimum response) to 4+ (maximum response) were assigned per crop sac and the rating per group calculated. The MED was the amount of pituitary tissue resulting in a minimal response in at least 3 of 5 crops.

The results of bioassays for MH activity in anterior pituitaries from normal and B_6-deficient rats are summarized in Table 2. The MED for crop stimulation in the test squabs was the same for normal and B_6-deficient rats, namely 1/32 gland (Table 3). However, the crop response obtained with injections of normal anterior pituitaries at most dosage levels were slightly better than the response obtained with B_6-deficient glands.

TABLE 3. THE GONADOTROPHIC ACTIVITY IN THE ANTERIOR PITUITARIES OF NORMAL AND OF B_6-DEFICIENT FEMALE RATS

Type of animal	FSH activity MED	ICSH activity MED	MH activity MED
	no. pits	no. pits	no. pits
Normal	5	1	$\frac{1}{32}$
B_6-deficient	$\frac{3}{4}$	$\frac{3}{4}$	$\frac{1}{32}$

DISCUSSION

The data presented show that the FSH activity of the B_6-deficient anterior pituitary was approximately 7 times higher per gland than that of the normal anterior pituitary, while the ICSH activity was only slightly higher. Moreover, the FSH activity of the B_6-deficient gland was approximately 10 times that of the normal gland when expressed per mg. tissue, inasmuch as the anterior pituitaries of B_6-deficient rats averaged $\frac{2}{3}$ the weight of normal glands. This increase in FSH activity of the pituitary might result either from (1) an increased rate of both production and release of FSH, such as occurs after oophorectomy, e.g., Evans and Simpson (1929), Martins and Rocha (1931) and Hill (1933); or (2) a normal rate of production of FSH associated with a decreased rate of release and consequent storage of the hormone; or (3) a decreased rate of production of FSH with defective or no release, resulting in the accumulation of abnormal quantities of the hormone over a period of time. The degree of follicular development in B_6-deficient ovaries indicated the presence of some circulating FSH, but in less than normal amounts, rather than the increased amounts that occur in the blood of castrate animals as shown by parabiotic studies (e.g. Martins and Rocha, 1931). The bioassays together with the histological findings here presented therefore suggest a decreased release of FSH from the B_6-deficient pituitary. However, an improper utilization of the circulating gonadotrophins by the ovary would also have resulted in the defective growth and maturation of follicles. In such a case, a high level of circulating FSH, if present, would be detected only by blood bioassays.

ICSH was present in the pituitaries of B_6-deficient animals in slightly increased amounts as compared with the pituitaries of normal rats, but, if released, was apparently not utilized properly by the ovary. Histological studies of ovaries from B_6-deficient rats showed that the interstitial tissue

was atrophic, though morphologically not identical with the "deficient" interstitial tissue as seen in the hyophysectomized rat. Such a status might result either from (1) a decrease in the level of circulating ICSH, resulting in only partial maintenance of the interstitial cells, or (2) improper utilization of the hormone, or (3) insufficient time for complete regression to the "deficient" state. There are criteria other than morphology of the interstitial cells for the presence or absence of circulating ICSH, i.e. the ability of minute quantities of ICSH, insufficient for interstitial cell repair, to promote estrin production by synergism with FSH (Evans *et al.*, 1939; Fraenkel-Conrat *et al.*, 1940). The uteri and vaginas of B_6-deficient rats, however, offered little evidence of stimulation by estrin although the ovaries showed evidence of FSH activity. It would appear that either the release or the utilization of ICSH was more severely affected than that of FSH.

No significant difference in the mammotrophic activity of normal anterior pituitaries and B_6-deficient glands was detected in this study. For functional evidence of mammotrophic activity the estrous rhythm of the rat may be inadequate as the role of MH in the cycle of the rat is controversial. The relation of B_6-deficiency to MH may be more clearly determined by studies during pregnancy or lactation.

This comparison of the gonadotrophic activity of normal and B_6-deficient pituitaries indicates that production or synthesis of the gonadotrophic hormones by the B_6-deficient glands was not impaired. The data suggest that interference with either the release or the utilization of FSH and ICSH occurred. Previous studies have indicated a decreased utilization of all three gonadotrophins by B_6-deficient animals, since injected gonadotrophins maintained pregnancy in only 25% of B_6-deficient animals in contrast with the 80–100% maintenance obtained with ovarian hormones (Nelson, Lyons and Evans, 1953). Because of the interrelationship of the gonadotrophins necessary for normal reproductive function, however, the possibility of a specific relation of B_6 to ICSH alone cannot be excluded.

There are no previous reports on the gonadotrophic content of pituitaries from B_6-deficient rats although studies have been made on the gonadotrophic potency of the pituitaries of rats subjected to other dietary deficiencies or to food restriction ("inanition"). In the study of any given dietary condition the findings have varied both with the methods of bioassay and with the severity of the deficiency, e.g. Werner (1939); Meites and Reed (1949); Drummond *et al.* (1939); and P'An *et al.* (1949). In a recent study of inanition Rinaldini (1949) has reported that the gonadotrophic potency of pituitaries from underfed female rats after a 35% loss in body weight was 3 times that of the normal gland, as judged by increased ovarian weights in hypophysectomized rats. Pearse and Rinaldini (1950) have reported that this increased gonadotrophic content could be demonstrated

histochemically. Such evidence suggests that although inanition may be a contributing factor, it could account for only part of the 7-fold increase in pituitary gonadotrophic potency observed in this study of B_6-deficient rats in which there was only a 10% loss in body weight. Both paired-feeding experiments and histological studies on B_6-deficient pituitaries are in progress in this laboratory.

SUMMARY

Assays for follicle stimulating, interstitial cell stimulating, and mammotrophic activity have been carried out on the anterior pituitaries from normal and B_6-deficient female rats, using immature hypophysectomized female rats and immature squabs as test animals. The anterior pituitary of the B_6-deficient rat showed 7 times as much FSH activity per gland as that of the normal female rat and 10 times the FSH activity per mg. of tissue. The ICSH activity was only slightly higher in B_6-deficient pituitaries and the MH activity was unchanged from that of normal glands.

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REFERENCES

Bahn, R. C., N. Lorenz, W. A. Bennett and A. Albert: *Proc. Soc. Exp. Biol. and Med.* **82**: 777. 1953.

Drummond, J. C., R. L. Noble and M. D. Wright: *J. Endocrin.* **1**: 275. 1939.

Evans, H. M. and M. E. Simpson: *Am. J. Physiol.* **89**: 371. 1929.

Evans, H. M., M. E. Simpson, S. Tolksdorf and H. Jensen: *Endocrinology* **25**: 529. 1939.

Evans, H. M., M. E. Simpson and W. R. Lyons: *Proc. Soc. Exp. Biol. and Med.* **46**: 586. 1941.

Fraenkel-Conrat, H., C. H. Li, M. E. Simpson and H. M. Evans: *Endocrinology* **27**: 793. 1940.

Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart: *J. Biol. Chem.* **138**: 459. 1941.

Hill, H. T.: *Endocrinology* **17**: 414. 1933.

Lyons, W. R.: *Proc. Soc. Exper. Biol. and Med.* **54**: 65. 1943.

Lyons, W. R. and E. Page: *Proc. Soc. Exper. Biol. and Med.* **32**: 1049. 1935.

Martins, T. and A. Rocha: *Endocrinology* **15**: 421. 1931.

Meites, J. and J. O. Reed: *Proc. Soc. Exp. Biol. and Med.* **70**: 513. 1949.

Nelson, M. M., W. R. Lyons and H. M. Evans: *Endocrinology* **48**: 726. 1951.

Nelson, M. M., W. R. Lyons and H. M. Evans: *Endocrinology* **52**: 585. 1953.

P'An, S. Y., H. B. Van Dyke, H. Kaunitz and C. A. Slanetz: *Proc. Soc. Exp. Biol. and Med.* **72**: 523. 1949.

Pearse, A. G. E. and L. M. Rinaldini: *Brit. J. Exper. Path.* **31**: 540. 1950.

Rinaldini, L. M.: *J. Endocrinol.* **6**: 54. 1949.

Werner, S. C.: *Proc. Soc. Exper. Biol. and Med.* **41**: 101. 1939.

THE QUANTITATIVE HISTOCHEMICAL DISTRIBUTION OF β-GLUCURONIDASE IN THE ADRENAL OF VARIOUS SPECIES AND THE INFLUENCE OF STRESS CONDITIONS, HORMONAL TREATMENTS AND HYPOPHYSECTOMY[1,2]

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AS A continuation of an earlier study by Wattenberg and Glick (1953) on the quantitative histological distribution of β-glucuronidase in the cow adrenal, the present investigation deals with the distribution of the enzyme in the adrenal of the Macacus rhesus and M. cynomolgus monkey, the rabbit, guinea pig, and rat. Peculiar species differences have been found.

Effects were studied of exposure of monkeys to combined hyperthermia and hypoxia, and of injections of ACTH, cortisone or desoxycorticosterone on adrenal β-glucuronidase activity. Observations were also made on the enzyme in the adrenals of monkeys with pulmonary lesions. Effects of hypophysectomy in the rat were likewise determined.

EXPERIMENTAL

Animals

The M. rhesus and M. cynomolgus monkeys used were those which had been employed for the study of the distribution of ascorbic acid in the adrenal (Bahn and Glick 1954). They were housed, fed, treated and killed as described previously.

Albino rabbits of both sexes were used, having body weights in the range of 3.4–4.5 kg., in addition to female guinea pigs weighing 500–900 gm., and albino male rats (Holtzman strain) weighing 400–500 gm. These small laboratory animals were caged in a room at 74 ± 2° F. All of the animals were fed Purina laboratory chow and tap water *ad libitum*, but the guinea pigs and rabbits were given cabbage as well. All animals were killed at 10 A.M. The rabbits were shot in the head in the same manner as described for the monkeys (Glick *et al.*, 1954a). The other species were killed by a single hammer blow

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[2] This study was supported by funds provided under contract AF 18(600)169 with the USAF School of Aviation Medicine, Randolph Field, Texas.

[3] Data in this paper were included in a thesis submitted in partial fulfillment of the requirements of the Graduate School of the University of Minnesota for the degree of Doctor of Philosophy.

on the head. A post mortem examination was carried out on all of the animals to observe whether signs of gross pathological changes were present. Only animals considered normal were used.

Unoperated, sham-operated and hypophysectomized male albino rats were obtained from the Hormone Assay Laboratories, Inc., Chicago. Initially all of the animals weighed about 300 gm. The sham-operated and hypophysectomized rats were sacrificed 6 days after operation and their weights were then 300–320 gm. and 240–250 gm. respectively. All of these animals were housed, fed, and cared for in the manner described for the other rats used in this study. Six animals were used in each group and the mean adrenal weights with standard errors of the mean were 23.8 ± 0.9 mg. (unoperated), 27.2 ± 1.9 mg. (sham-operated), and 10.1 ± 0.3 mg. (hypophysectomized).

Cow adrenals were also used to check previous work and these were obtained from animals immediately after killing at a local meat packing house.

Sampling and Histological Preparation

The rapid removal of the adrenals from the animals, the quick freezing, sampling, sectioning of the fresh frozen tissue, and histological preparation and staining were conducted as described earlier by Bahn and Glick (1954) and Glick *et al.* (1954b). For each of the serial sections which was employed for enzyme analysis the previous adjacent section was used for histological identification after staining with toluidin blue, and the following adjacent section was used for protein analysis.

Chemical Analysis

The equipment and reagents used for the determination of β-glucuronidase activity using phenolphthalein mono-β-glucuronide as substrate were the same as those employed in the earlier work of Wattenberg and Glick (1953). In this study the results were expressed as activity per μg. of protein-nitrogen which was estimated by the bromsulfalein procedure (Nayyar and Glick 1954). In the case of sections of rat adrenal, which constitute samples smaller than usual, the concentration of bromsulfalein in the bromsulfalein reagent was reduced to half. The volume of the reagent was not changed. The concentration of the sodium hydroxide used for the development of the final color was increased to 0.2 N and the volume employed was reduced to 0.5 ml.

The protein-nitrogen was measured on individual tissue sections rather than on the tissue material used in each enzyme analysis. Admittedly, it would be preferable to measure the protein actually present in each enzyme reaction mixture, but this would entail the precipitation of the protein, removal of the supernatant for the glucuronidase assay, and measurement of the protein in the precipitate. It has been reported that trichloracetic precipitates of protein, such as those obtained in the procedure of Fishman *et al.* (1948) for the estimation of glucuronidase, adsorb the phenolphthalein to some degree (Mills *et al.*, 1953; Lorincz *et al.*, 1951; and Moore *et al.*, 1950). Control experiments carried out in this study revealed that about 15% of the phenolphthalein liberated by the enzyme action was adsorbed on both trichloracetic and tungstic acid precipitates of the protein. Therefore it was considered undesirable to use the protein in the enzyme reaction mixture for the analysis.

The procedure used for the β-glucuronidase determination differed from that previously published (Wattenberg and Glick, 1953) only in a few details. In the present instance a volume of 9.4 μl. of the 30% glycerol was used to extract the enzyme, 1 hour was allowed for the extraction, and 9.4 μl. of buffer-substrate solution was used. The digestion period was varied from 30 minutes to 2 hours, depending on the extent of the

enzyme action, and 18.2 μl. of glycine buffer was used to stop the reaction. When high enzyme activities resulted in the production of a solution which was too intensely colored for accurate measurement (optimum accuracy is obtained when the optical density is confined to the range 0.2–0.7, Ayers, 1949) greater known volumes of glycine buffer were used to lower the optical density below 0.7. In the experiments reported the total hydrolysis of substrate never exceeded 15% of that present, and the change in substrate concentration which was produced was not sufficient to affect the rate of the enzymatic action.

The data obtained were represented in composite curves giving chemical values as a function of distance through the adrenal. These curves were constructed in the fashion described by Bahn and Glick (1953).

The use of fresh tissue involves the question of whether the blood present contributes appreciably to the β-glucuronidase activity measured. To clarify this point control experiments were carried out on whole blood and serum and the enzyme activities observed were negligible (<1%) compared to those of the tissue samples. Hemoglobin color interferes with the measurement of the phenolphthalein color and therefore, in whole blood analysis, precipitation with trichloracetic acid was employed. The loss of phenolphthalein by adsorption on the precipitate, as noted earlier in this paper, renders the values too low but, even if a generous allowance is made for this factor, the values are not significant. Earlier data indicate that red blood cells and platelets contain very little of the activity of the whole blood, but that the leucocytes and lymphocytes do possess a major portion of this activity (Fishman *et al.*, 1948; Rossiter and Wong, 1950a, b).

Becker and Friedenwald (1949) demonstrated that ascorbic acid, *in vitro*, inhibits β-glucuronidase. Under the conditions they used, inhibitions of 83, 22, and 0% of rat liver enzyme were effected by 20, 2, and 0.2 mg.% ascorbic acid, respectively. A calculation from the data of Bahn and Glick (1954) of the concentrations of ascorbic acid, which are supplied by the adrenal sections, in the reaction mixtures used for the present β-glucuronidase determinations reveals a maximum of about 1 mg.% for the rat tissue with a minimum of about 0.4 mg.%. The corresponding values for the monkey tissue are about 0.4 and 0.2 mg.%. This suggests that the naturally occurring ascorbic acid in adrenal tissue probably has no very great effect on the β-glucuronidase assays.

Stability of β-glucuronidase in the frozen adrenal

To determine whether storage in the frozen state affects the β-glucuronidase activity of the adrenal, an experiment was conducted in which samples were taken from a cow adrenal 4 hours, 1, 2, and 7 days after the initial removal of the gland from the animal, its freezing with solid carbon dioxide, and storage at −15° C. The data given in Figure 2 show that the enzyme activity was not significantly changed even after 7 days of storage at this temperature. In several instances monkey adrenals were stored at −15° C. up to 12 hours prior to the time of sectioning and analysis, and the data obtained did not differ significantly from those found with adrenals which were analyzed immediately after removal from the animals.

RESULTS

The composite curves illustrating the distribution of β-glucuronidase activity in the adrenals of the guinea pig and rat are given in Figure 1. In both species the activities are of similar magnitude and the maximum ac-

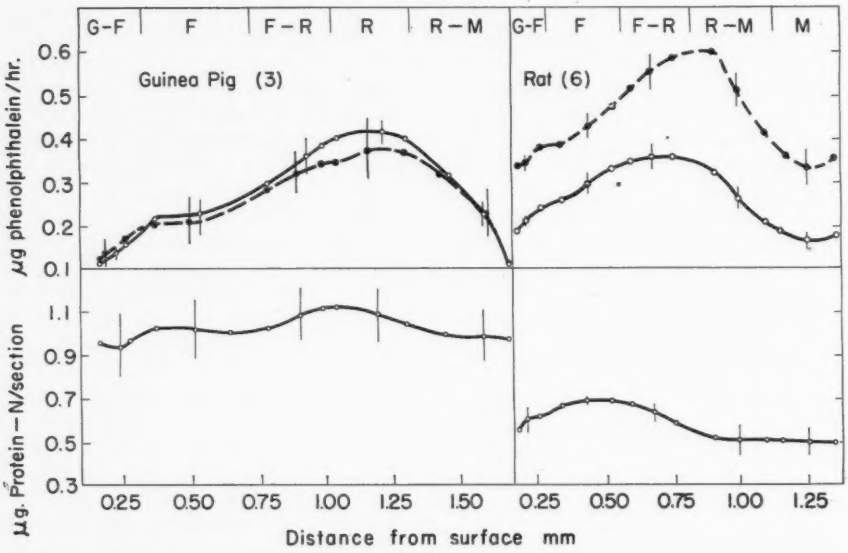

FIG. 1. Distribution of β-glucuronidase activity and protein-nitrogen in the adrenal glands of the female guinea pig and albino male rat. Microtome sections of the guinea pig adrenals were 2 mm. in diameter, 16 μ thick (0.05 μl. vol.), those of the rat adrenals were 1.5 mm. in diameter, 16 μ thick (0.028 μl. vol.). In all of the figures the following apply: enzyme activity per section upper solid lines; activity per μg. protein-nitrogen broken lines, the ratios of upper to lower solid lines; points represent means, vertical lines through points the standard error of means. Histological zones are indicated by C (capsule), G (glomerulosa), F (fasciculata), R (reticularis), M (medulla). When sections are mixtures of 2 zones both designating letters are used, *e.g.* F-R. The numbers in parentheses after the name of the species denote the number of adrenals analyzed to obtain the composite curves.

tivity occurs in the reticular zone with a falling off to the medulla on the one side and through the fasciculata to the glomerulosa on the other. It was not possible to obtain sections of pure reticularis in the smaller rat adrenal, but as the proportion of reticular material increased in the sections, the activity rose correspondingly to make it clear that the greatest activity was associated with this zone.

In the earlier report of Wattenberg and Glick (1953) on the cow adrenal a peak of activity was found in the glomerular zone with decreasing activities through the other cortical zones to a minimum in the medulla. In view of the differences which were subsequently found in the distribution of the enzyme in the adrenal of other species, the study on the cow adrenal was repeated and the results (Fig. 2) confirmed the earlier report.

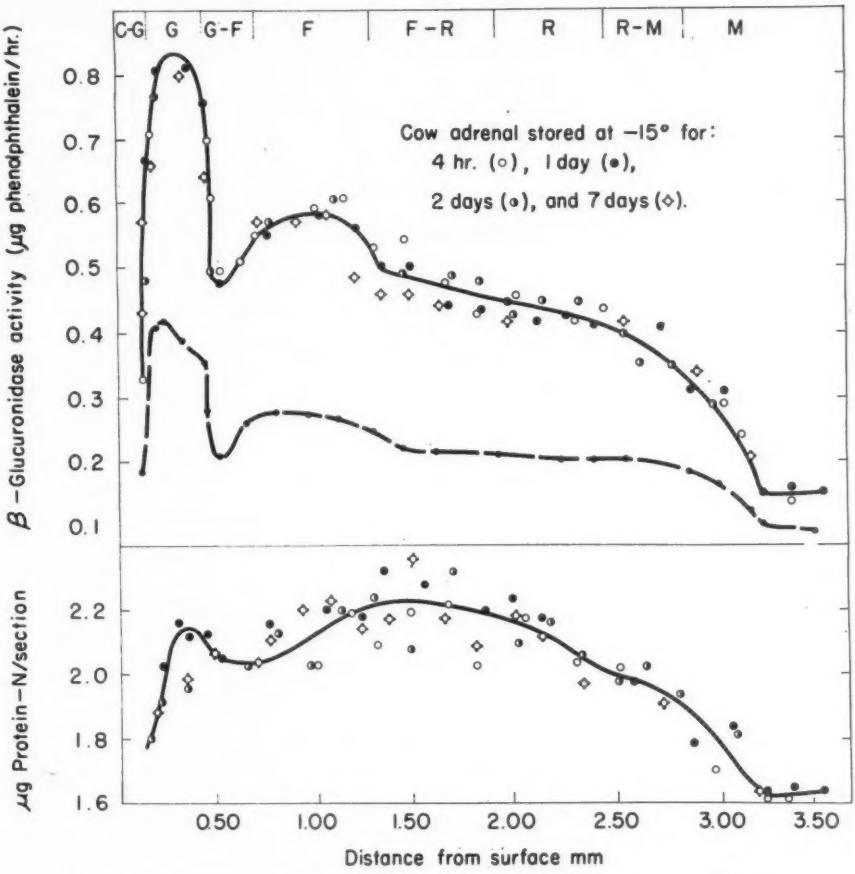

FIG. 2. Distribution of β-glucuronidase activity and protein-nitrogen in the cow adrenal. Sections 3 mm. in diameter, 16 μ thick (0.113 μl. vol.).

Data for the rabbit adrenal (Fig. 3) show very little activity in any region, thus emphasizing the great species variability in the activity of this enzyme.

In the adrenals of M. rhesus and cynomolgus monkeys (left half, Figs. 4, 7) the profile of the distribution of the activity may be seen to correspond to that in the guinea pig and rat adrenal with the reticular maximum. The peak activity in the rhesus adrenal is somewhat greater than that in the cynomolgus organ and the latter is of the same order as that found in the adrenals of the guinea pig and rat.

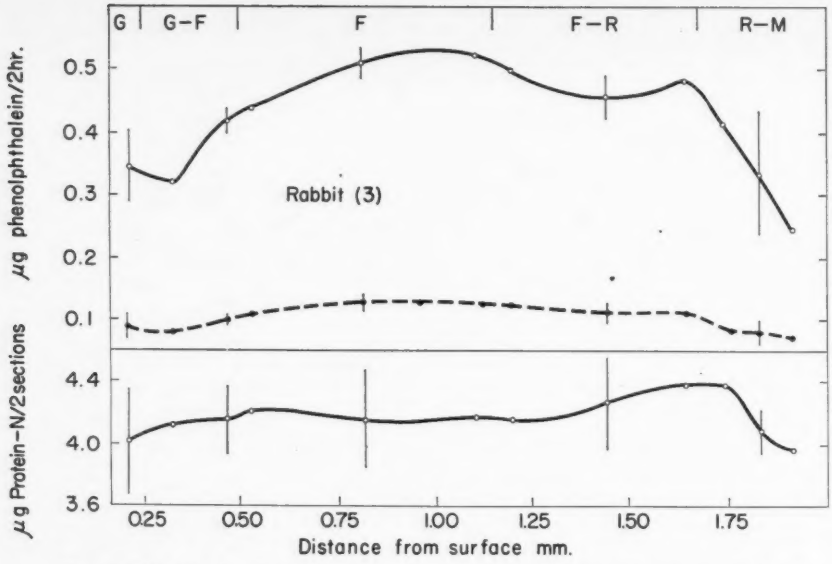

FIG. 3. Distribution of β-glucuronidase activity and protein-nitrogen in the rabbit adrenal. Sections 3 mm. in diameter, 16 μ thick (0.113 vol.).

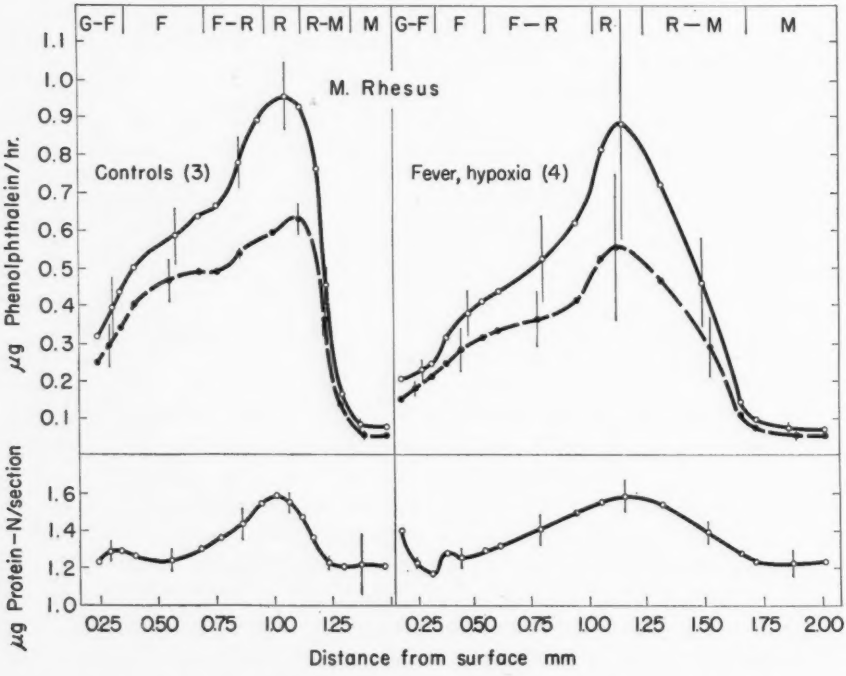

FIG. 4. Distribution of β-glucuronidase activity and protein-nitrogen in the adrenal gland of M. rhesus monkeys with and without combined fever and hypoxia. Sections 2.5 mm. in diameter, 16 μ thick (0.079 μl. vol.).

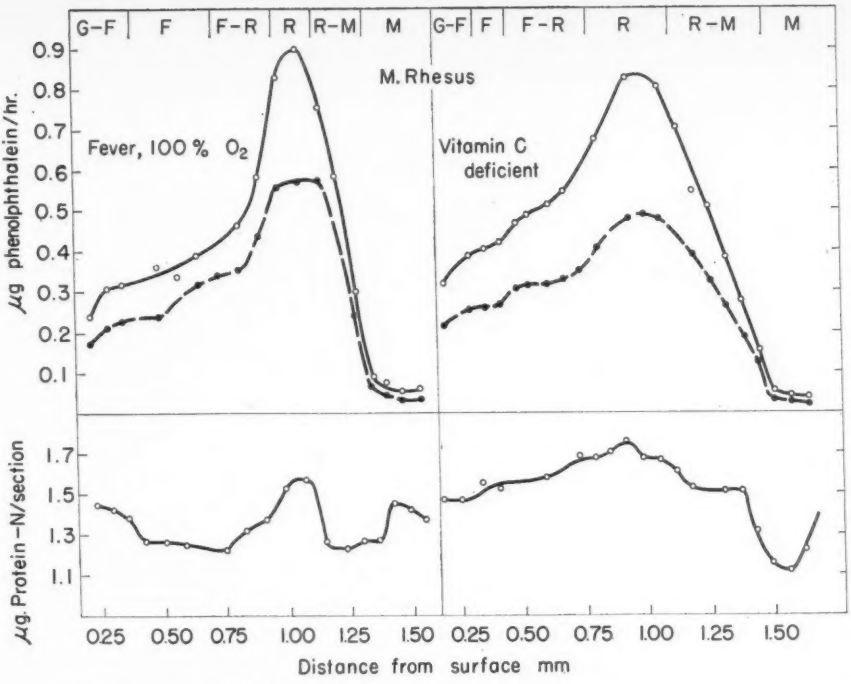

FIG. 5. Distribution of β-glucuronidase activity and protein-nitrogen in the M. rhesus monkey adrenal following combined fever and oxygen administration and vitamin C deficiency. Sections 2.5 mm. in diameter, 16 μ thick (0.079 μl. vol.).

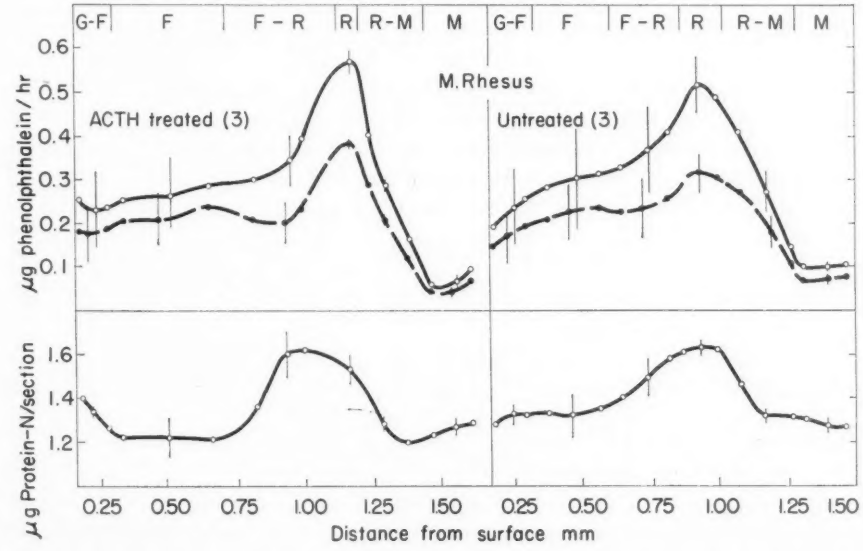

FIG. 6. Distribution of β-glucuronidase activity and protein-nitrogen in the adrenals of M. rhesus monkeys with pulmonary granulomas, with and without single intraperitoneal injections of 15 mg. ACTH per kg. body wt. 3 hr. prior to sacrifice. Sections 2.5 mm. in diameter, 16 μ thick (0.079 μl. vol.).

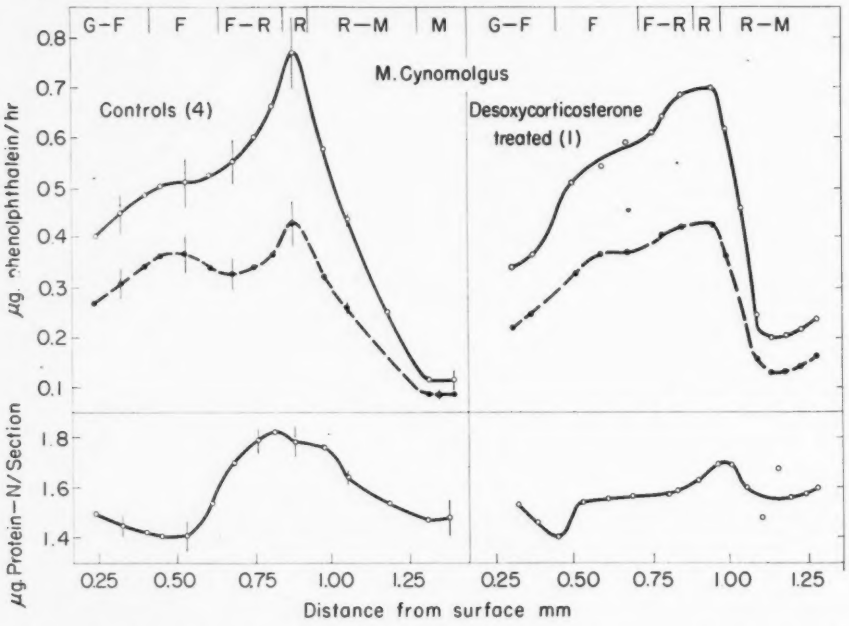

FIG. 7. Distribution of β-glucuronidase activity and protein-nitrogen in the adrenals of M. cynomolgus monkeys with and without single intramuscular injections of 10 mg. desoxycorticosterone acetate per kg. body wt. 24 hr. prior to sacrifice. Sections 2.5 mm. in diameter, 16 μ thick (0.079 μl. vol.).

Although great individual variation occurred, the combined fever and hypoxia did not significantly change the distribution or magnitude of the activity in the rhesus adrenal (Fig. 4). This was also true for the single cases of fever with oxygen administration and vitamin C deficiency (Fig. 5). The same general distribution profile with lower activities was observed when rheuss monkeys with pulmonary granulomas, either untreated or treated with ACTH, were employed (Fig. 6). In the cynomolgus strain treatment with desoxycorticosterone (Fig. 7), ACTH or cortisone (Fig. 8) had no significant influence.

In adrenals of hypophysectomized rats increased activities are seen in all zones (Fig. 9). Adrenals of sham-operated rats had a tendency to lower activities which does not appear to be significant.

DISCUSSION

The striking qualitative and quantitative differences which have been observed in the β-glucuronidase activities of the various histological zones of the adrenal in the different species are not understood at present. Pos-

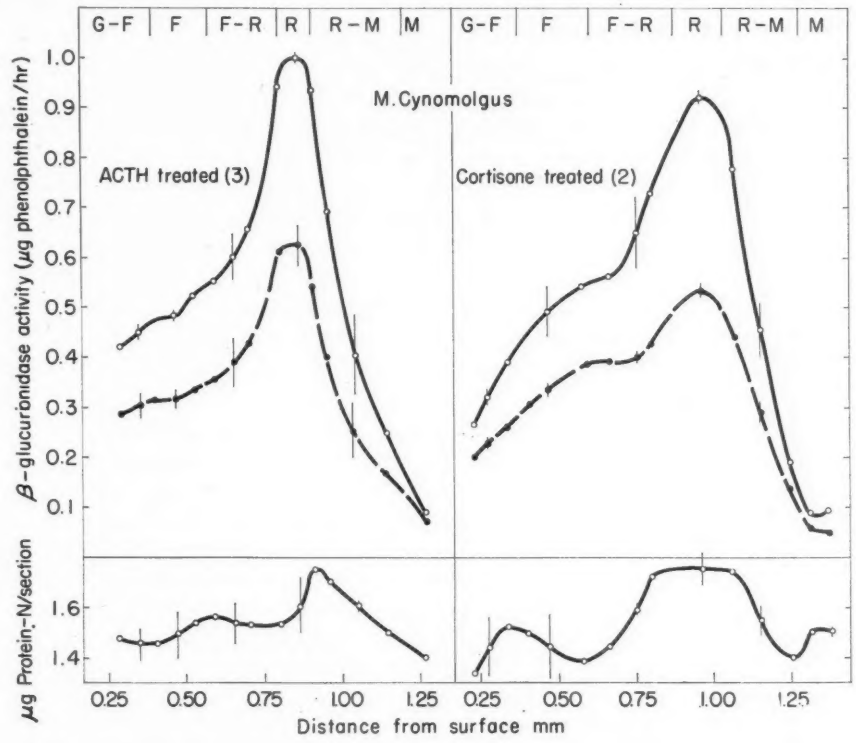

FIG. 8. Distribution of β-glucuronidase activity and protein-nitrogen in the adrenals of M. cynomolgus monkeys given single intraperitoneal injections of 30 mg. ACTH per kg. body wt. 3 hr. prior to sacrifice, or single intramuscular injections of 10 mg. cortisone acetate per kg. body wt. 24 hr. prior to sacrifice. Sections 2.5 mm. in diameter, 16 μ thick (0.079 μl. vol.).

sible correlations with functional differences among these species should be investigated. In this connection the report of Bush (1953) should be cited in which differences in adrenal hormone secretion among certain species were revealed. The ratio of 17-hydroxycorticosterone to corticosterone secreted was <0.05 for the rat, <0.10 for the rabbit, and 20 for the rhesus monkey. In the adrenal secretion of the rat, but not in that of the rabbit or monkey, 11-hydroxyandrostenedione was found. The maximal secretory rates of Δ^4-3-ketosteroids fell in the order rat > monkey > rabbit. No obvious correlation can be drawn at present between these data and the species differences observed in the β-glucuronidase activities.

It is noteworthy that combined fever and hypoxia severe enough to cause death in rhesus monkeys had no appreciable effect on the distribu-

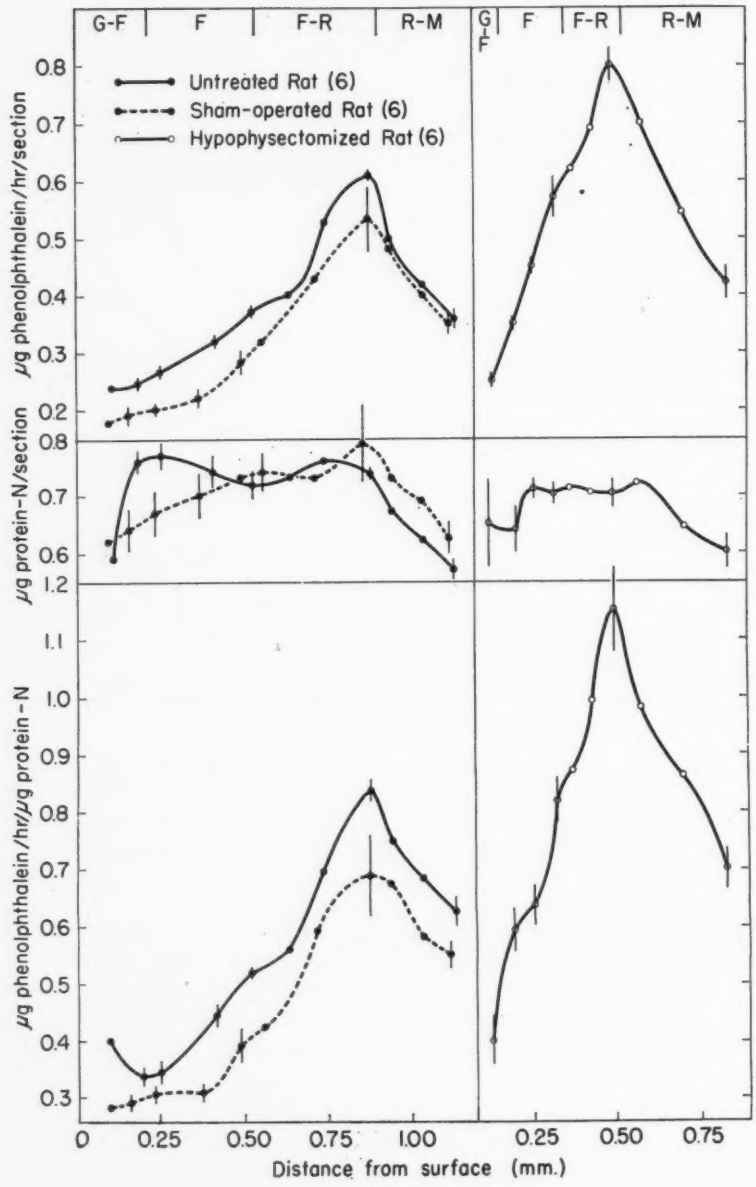

FIG. 9. Distribution of β-glucuronidase activity and protein-nitrogen in the adrenals of male albino rats; effect of hypophysectomy and sham-operation. The treated animals were sacrificed 6 days after operation. Sections 1.5 mm. in diameter, 16 μ thick (0.028 μl. vol.).

tion or magnitude of the β-glucuronidase activity in the adrenal. This also applied to the animal which succumbed to fever while being supplied with oxygen, to the animal suffering from a vitamin C deficiency which produced no obvious clinical symptoms but which caused the concentration of the vitamin in the adrenal to fall drastically, and to those, with and without ACTH treatment, which had pulmonary granulomas. Furthermore, no effect on the activity can be ascribed to the treatments of cynomolgus monkeys with ACTH, cortisone, or desoxycorticosterone. Cohen (1951) reported an increase in β-glucuronidase activity in human blood serum after cortisone treatment.

Data in this paper are in accord with those of Knobil (1953) who found that no change in β-glucuronidase activity per unit wet weight of whole rat adrenal occurred after subcutaneous injections of ACTH (2 mg./day for 4 or 10 days) or cortisone (3 mg./day for 10 days), which were sufficient to cause adrenal hypertrophy and atrophy, respectively. Furthermore, 6 days after hypophysectomy the concentration of the enzyme in whole adrenals was significantly greater than in the controls. If the total activity per adrenal is calculated from the data of Knobil, it may be seen that the ACTH treatments elicited an increase of about 23 and 42% for the 4 and 10 day treatments, respectively (corresponding adrenal weights increased 28 and 38%); the cortisone injection caused a decrease of about 45% (adrenal weight decrease, 51%), and hypophysectomy effected decreases of about 37 and 29% in 2 different experiments (corresponding adrenal weight decreases, 45% in both cases). It follows that the hypertrophy from ACTH resulted in an increase in the enzyme activity which was approximately proportional to the increase in the adrenal weight, and the atrophy from cortisone caused a decrease in the activity which was approximately proportional to the decrease in the adrenal weight. Thus it is clear that the activity per unit of cell substance under these 2 conditions was rather constant, and that the activity per hypertrophic cell was greater, and that of the atrophic cell less, than the normal. However, the atrophy from hypophysectomy caused a decrease in activity which was less than the adrenal weight decrease in one case but not very different in the other, so that an actual increase in enzyme concentration and enzyme content per cell occurred in at least one of the two experiments. While the data given in the present report support the results of Knobil with respect to effects on enzyme concentration in the tissue, in the absence of data on cell number per unit volume of tissue before and after the treatments, it is not possible to ascertain effects on the enzyme content per cell in our material.

SUMMARY

The quantitative histological distributions of β-glucuronidase activity in the adrenal gland of the M. rhesus and M. cynomolgus monkey, cow,

rabbit, guinea pig and rat have been determined, and peculiar species differences have been found.

Exposure of monkeys to combined fever and hypoxia to the point of death, and treatment with ACTH, cortisone or desoxycorticosterone had no significant influence on the distribution or magnitude of the concentration of β-glucuronidase activity in the adrenal.

Hypophysectomy in rats increased the activity in all zones of the adrenal when expressed either as activity per unit volume of fresh tissue or per unit protein-nitrogen.

ADDENDUM

The role of β-glucuronidase and of sulfatase in the adrenal gland remains obscure, but we should like to suggest the following working hypothesis in the hope of stimulating thought and work on the problem. As has been mentioned by Glick and Stecklein (1954) in a consideration of phenolsulfatase in the adrenal, this gland may be involved in supplying free hormone, in response to physiological demand, from the blood pool of conjugate which is brought to it. The maintenance of the physiological level of hormone conjugate in the blood stream is kept by the balance between production, primarily by the liver, excretion, primarily by the kidney, and breakdown in tissues and body fluids. In addition to the direct formation of hormones by the adrenal, which has been well established, a secondary mechanism may exist which permits the use of hormone present as conjugate in the blood stream when the need for a supplemental source arises. The increase in β-glucuronidase activity after hypophysectomy might then be a compensatory effect by which the decreased production of hormones by the adrenal is offset to some degree by the increased enzymatic liberation of the hormone from the pool of conjugate previously formed. One might expect a similar effect from inhibition of adrenal activity following administration of cortisone. However, in the work reported here only single injections of the hormones were given and it would be necessary to administer the hormones repeatedly to inhibit the adrenal to a degree comparable with hypophysectomy.

REFERENCES

Ayers, G. H.: *Anal. Chem.* **21**: 652. 1949.
Bahn, R. C. and D. Glick: *J. Histochem. Cytochem.* **2**: 103. 1954.
Becker, B. and J. S. Friedenwald: *Arch. Biochem.* **22**: 101. 1949.
Bush, I. E.: *J. Endocrinology* **9**: 95. 1953.
Cohen, S. L.: *Ann. N. Y. Acad. Sci.* **54**: 558. 1951.
Fishman, W. H., B. Springer and R. Brunetti: *J. Biol. Chem.* **173**: 449. 1948.
Glick, D., and H. R. Stecklein: *Arch. Biochem. Biophys.*, in press, 1954.
Glick, D., R. H. Swigart, R. C. Bahn and D. W. Hastings: *Endocrinology*, **54**: 706. 1954a.

GLICK, D., R. H. SWIGART, S. N. NAYYAR AND H. R. STECKLEIN: *J. Histochem. Cytochem.*, in press. 1954b.
KNOBIL, E.: *Proc. Soc. Exper. Biol. Med.* **83**: 769. 1953.
LORINCZ, A. L., J. NOVELLI, L. S. MCGOOGAN AND L. D. ODELL: *Am. J. Obstet. Gyn.* **61**: 527. 1951.
MILLS, G. T., J. PAUL AND E. E. B. SMITH: *Biochem. J.* **53**: 232. 1953.
MOORE, W. R., E. S. COHEN AND M. MCDONALD: *Harper Hosp. Bull.* **8**: 141. 1950.
NAYYAR, S. N. AND D. GLICK: *J. Histochem. Cytochem.*, **2**: 282. 1954.
ROSSITER, R. J. AND E. WONG: *Blood* **5**: 864. 1950a.
ROSSITER, R. J. AND E. WONG: *Can. J. Res.* **28E**: 69. 1950b.
WATTENBERG, L. W. AND D. GLICK: *J. Histochem. Cytochem.* **1**: 1. 1953.

CORTISONE AND PHAGOCYTOSIS

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AS HAS been pointed out by Thomas (1952, 1953), cortisone has been demonstrated to reduce the resistance of mammals to bacteria, viruses, fungi, and bacterial toxins. In discussing the various methods whereby cortisone might act to produce these results, the author suggests the conclusion that the effect must be on the reticuloendothelial system (RES). At about the same time, it was pointed out that cortisone appeared to depress several functions of the RES if given in sufficient dosages (Heller, 1953). This paper concerns itself with further investigation into the specific nature of the effect of cortisone on RES functions involved in infection.

MATERIALS AND METHODS

Relying on the exclusive ability of the RES to take up negatively charged colloids (Goulian, 1953), radio-active colloidal chromium phosphate labeled with P^{32} was prepared for use as an indicator according to the technique of Jones (1944). Heterogeneity of particle size (demonstrated by electron micrography) was deliberately sought because the RE cells of various organs selectively phagocytize particles of different size range (Gabrieli, 1952). Amounts of this colloidal chromium phosphate equal to approximately 10 micrograms of solid material and 1 to 10 microcuries of P^{32} were suspended in glucose solution and injected intravenously in mice and rats. Within 3 to 4 minutes 6 blood samples were drawn, each sample containing 5 lambdas. Each one was placed on a one-inch filter paper disc and measured for radioactivity. Radioactivity was plotted against time. As shown in Figure 1, a strictly exponential function obtains. By measuring the slope of the curve, the intravascular half-life of the colloid can be determined. From 5 to 7 animals were used to establish a single point, and no data were accepted unless the p value was less than .01. This method provides an index of the number and/or rate of activity of phagocytizing RE cells.

In order to render the tests of phagocytic function of the RES more sensitive, an injection of Thorotrast (thorium dioxide)[1] was given 24 hours prior to the injection of $CrP^{32}O_4$. Measurement of residual blockade by this substance at the end of this period gives an indication of the vitality of the recuperative process.

Cortisone (Cortone, Merck) was used and diluted with saline when necessary. It was

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[1] Kindly supplied by Fine Chemicals Division of the American Cyanamid Company, Princeton, New Jersey.

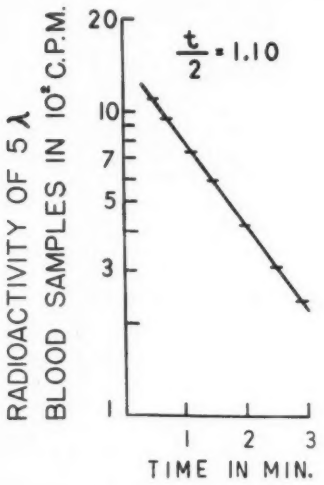

FIG. 1. The curve of phagocytic velocity obtained by plotting the radioactivity of blood samples against time.

given intramuscularly for 3 days; the dosages varying betweeen 0.25 and 5 mg. per 20 gm. mouse and 5 mg. to 20 mg. per 140 gm. rat. The suspending vehicle[2] served initially as a control. No significant difference was found between it and saline.

A total of 160 Wistar strain albino male rats weighing 125 to 150 gm. were used. However, in each experiment no more than a 10 gram variation in experimental and control rats was permitted. A total of 150 ninety day old DBA mice were used. Only males were tested, since females show cyclic variation in phagocytic activity at different phases in their estrus cycle. The mice were kept in individual cages so as to preclude the possibility of fighting. Standard laboratory chow was fed

RESULTS

In the rat experiments it can be seen that the intravascular half-life (t/2) and hence the phagocytic velocity of the injected radio-colloid is significantly lengthened as a function of cortisone administration (Fig. 2).

The ability of the RES to recover from the effect of a blockade by Thorotrast was tested in the next series of experiments. In order to determine the ability of the RE cells to regenerate, or to recover phagocytic function after a Thorotrast blockade, it was shown that cortisone markedly depressed this regenerative capacity, as can be seen in Figure 3. Here the inability of the cortisone-treated animals to cope with increasing amounts of intravascular colloid after 24 hours is in marked contrast to the control series of animals.

Essentially, the results with mice were the same as with rats. Since it was

[2] Kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

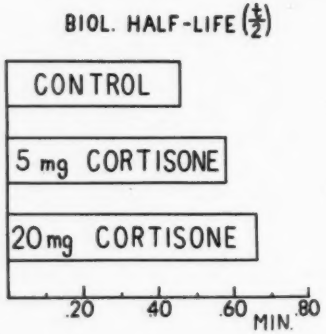

FIG. 2. The increase in intravascular half-life of $CrP^{32}O_4$ after cortisone administration. Twenty animals are used in each group. Statistical analysis shows the difference to be significant with $p < .05$ for the 5 mg. dose and $p < .01$ for the 20 mg. dose.

apparent that relatively large doses of cortisone were necessary in order for this method to show RE depression, it was decided to devise a way whereby effects might be demonstrated with smaller amounts. To accomplish this end, a partial blockade was produced in the animals 24 hours before the administration of $CrP^{32}O_4$ by an intravenous injection of Thorotrast amounting to .10 or .15 cc. per 100 gm. body weight. Figure 4 shows the mean intravascular half-life (t/2) of chromium phosphate in

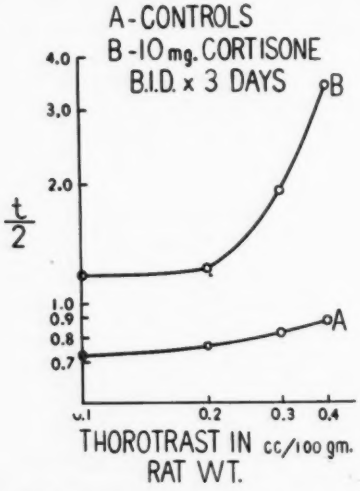

FIG. 3. Measurement of the regenerative capacity of phagocytic function 24 hours after varying doses of Thorotrast. Ordinate: Intravascular half-life (t/2) in minutes. Abscissa: Thorotrast dosage. Each point represents 8 animals. Statistical analysis shows the difference to be highly significant with $p < .001$.

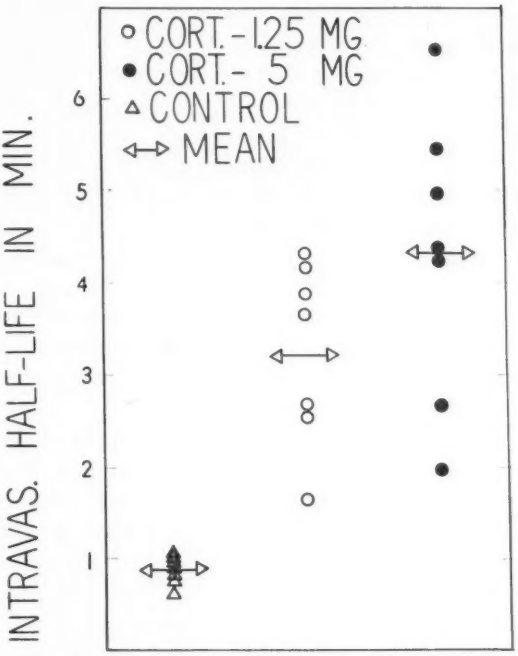

FIG. 4. The spread in the mean of individual values of the intravascular half-life (t/2) of $CrP^{32}O_4$ in normal mice versus mice treated with different amounts of cortisone.

two series of mice given 1.25 mg. and 5 mg. of cortisone per animal, respectively. Depression of RE function could be demonstrated with cortisone administration down to a dosage of 0.25 mg. per animal (significant at the 5% level).

Finally, radioautographs of the animals' livers and spleens showed markedly different distribution patterns of the radioactive colloid in the cortisone-treated animals as opposed to the controls. These data will be presented elsewhere.

DISCUSSION

Supporting the conclusion which Thomas (1953) reached, it appears that, with an accurate and sensitive, reproducible method of measuring certain functions of the RES, there is little question that cortisone causes a depression of phagocytic RE function. There have been several attempts by various authors to evaluate the effect of cortisone on the phagocytic function of the RES—Gordon and Katsh (1949), Lurie *et al.* (1951), Spain *et al.* (1950), Friebel (1952), Clawson (1953), Teodoru *et al.* (1952),

and Benacerraf *et al.* (1954). Inconsistent results were obtained, varying from conclusions that phagocytosis is markedly reduced, is markedly increased, or that it is unchanged except for the fact that there is an inability of the macrophages to handle the phagocytosed bacteria.

It should be pointed out that the predominant method of measurement in most of these papers has been morphological or has involved uptake of colloids by a single organ. It is virtually impossible to measure by such methods the total activity of the RES throughout the body, because it is so diffusely spread. At best, an impression may be obtained of the status of phagocytosis in the liver or spleen, or other isolated organs. Repeated experiments in this laboratory have demonstrated that it is perfectly possible to have an increased uptake by RE cells in one or more organs, and yet have the total phagocytic ability of the entire RES depressed. These data will be presented elsewhere.

Attempts to measure the phagocytic activity of the entire RES by India ink or vital stains have the limitations of the fact that most of them, i.e., India ink, have been demonstrated to be cytotoxic—Zilversmit (1954)—and can actually induce irritative hyperfunction of the RES. The use of heterogeneous particle size radiocolloid permits a far more accurate quantitation of phagocytic activity than do morphologic methods or the use of the India ink content of the liver and/or spleen.

The data presented above indicating the depression of the phagocytic function of the RES by cortisone help in understanding the results obtained by various authors such as Jawetz (1953), Ilavsky and Foley (1953), and Heller (1954). These authors have shown that cortisone increases mortality significantly in infection, even in spite of antibiotic therapy. In these studies the problem of antibody formation was of relatively little importance, because most of the mortality among the animals was within a 5 day period.

In addition to these animal data, the observation in humans of bacteremia following cortisone administration tends to support these findings. The problem as to what degree this depression of phagocytic function can be extrapolated to all other RE functions (such as antibody formation, etc.) is a matter that only further work will be able to determine. These experiments are now in progress.

SUMMARY

By the use of negatively charged colloidal $CrP^{32}O_4$ it is possible to measure certain functions of the RES. Using this method of measurement, it appears that cortisone depresses the ability of the RE cells to phagocytize and regenerate.

Partial Thorotrast blockade is used to increase the sensitivity of quanti-

tation. Functional depression of RE cells can be demonstrated with as little as 2.5 mg. of cortisone per kilo in mice. Both rats and mice showed essentially the same type of RES depression.

Acknowledgments

The author gratefully acknowledges the technical assistance of Miss Lorraine Barnett. This work was supported by a grant from the Schick and Luria Foundations.

REFERENCES

BENACERRAF, B., B. N. HALPERN, G. BIOZZI AND S. A. BENOS: *Brit. J. Exper. Path.* **35**: 97. 1954.

CLAWSON, B. J.: *J. Lab. Clin. Med.* **42**: 746. 1953.

FRIEBEL, H.: *Naunym-Schmiedeberg's Archiv fur Exper. Path. und Pharmak.* **216**: 506. 1952.

GABRIELI, E. R.: *Acta Path. Microbiol. Scand.* **31**: 195. 1952.

GORDON, A. S. AND G. F. KATSH: *Ann. N. Y. Acad. Sci.* **52**: 1. 1949.

GOULIAN, D.: *Federation Proc.* **12**: 211. 1953.

HELLER, J. H.: *Federation Proc.* **12**: 65. 1953.

ILAVSKY, J. AND E. J. FOLEY: *Proc. Soc. Exper. Biol. and Med.* **84**: 211. 1953.

JAWETZ, E. AND E. R. MERRILL: *Science.* **118**: 549. 1953.

JONES, H. B., C. J. WROBEL AND W. R. LYONS: *J. Clin. Invest.* **23**: 783. 1944.

LURIE, M. B., P. ZAPPASODI, A. M. DANNENBERG, JR. AND I. B. SWARTZ: *Science.* **113**: 234. 1951.

SPAIN, D. M., N. MOLOMUT AND A. HABER: *Science.* **112**: 335. 1950.

TEODORU, C., T. FEYEL-CABANES AND R. JEQUIER: *Arch. Int. Pharmacodyn.* **91**: 151. 1952.

THOMAS, L.: *Ann. Rev. Med.* **3**: 1. 1952.

THOMAS, L.: *Ann. N. Y. Acad. Sci.* **56**: 799. 1953.

ZILVERSMIT, D. B., M. L. SHORE AND C. H. GANTT: *Federation Proc.* **113**: 170. 1954.

THYROIDAL FUNCTION IN THE FISHES *FUNDULUS HETEROCLITUS F. MAJALIS*, AND *F. DIAPHANUS*[1]

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IN THE evolution of organs or systems it is generally believed that successive changes in them persist principally because they endow the organism with new properties important in the adaptability and continuance of the organism and species. In this sense the lack of what would appear to be an important function for the thyroid gland in fish presents a real challenge to the comparative endocrinologist. There is no clear indication of the nature of the value of the thyroid gland to the most primitive of living vertebrate groups that would have led to the evolution of this fairly complex gland as well as its system of functional control by the pituitary. It has been shown that the thyroid thickens the epidermis (Harms, 1929; LaRoche, 1952), may affect the pigmentary pattern (LaRoche, 1952; Robertson, 1948) and increase the retrobulbar connective tissue in various species of fish (LaRoche, 1952). Claims have been made that the piscine thyroid may be involved in oxidative metabolism (Smith and Matthews, 1948), or metabolism of salts and water ("osmoregulation") (Koch and Heuts, 1942), but little direct evidence exists to establish either possibility. This question recently was reviewed by Hoar *et al.* (1951). An extensive literature exists that denies that the thyroid hormone in fish is involved in oxidative metabolism (Hoar *et al.*, 1951).

If the thyroid of fish is involved in osmoregulation, as has been claimed by Koch and Heuts (1942), then its function may be important in the tolerance of different species for waters of different salinities. This, in turn might account for the ecological distribution of these species. The only other direct work in this question known to us is that of Olivereau (1948) indicating thyroidal stimulation follows when a marine species is moved into less saline water. For further examination of this question we undertook the study of thyroid function of three fairly closely related minnows of the genus *Fundulus*. *Fundulus majalis* normally lives in sea water; *Fundulus heteroclitus* inhabits brackish water; *Fundulus diaphanus* is a

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freshwater species. In these studies, thyroid function was measured in animals kept during an experimental period in water either more or less saline than their normal environment. A further variable, temperature, also was studied in regard to its influence on quantitative as well as qualitative features of thyroid function.

METHODS AND MATERIALS

The *F. diaphanus* specimens were collected in a Lake Michigan shore pool at Waugoshance Point near Cheboygan, Michigan. They were kept in running well water at 15° C. and were unfed during the period of experimentation. The *F. heteroclitus* and *F. majalis* were collected in a tidal inlet, the Wading River, on Long Island, New York. They were kept unfed in aquaria in aerated water taken from the Wading River at either of two tide intervals. The water was changed at approximately daily intervals. The laboratory temperature was kept at 22° C. High-tide water was closest to true sea water; it had a salinity of 25.5 parts per thousand expressed as equivalents of sodium chloride and a total iodine concentration of 43.7 μg. per liter.* Low-tide water was relatively fresh; it had a salinity of only 5.1 parts per thousand and its iodine concentration was 25.7 μg. per liter. The salinity of the Michigan lake water was not determined; its iodine concentration was 1.5 μg. per liter.

Radioiodine was injected intraperitoneally as carrier-free iodide. Animals were killed in groups of 4 to 6 for each point on the curves of thyroidal I^{131}-uptake, or I^{131}-chromatography. In the study of quantitative thyroidal uptake of I^{131} the lower jaws of the fishes, containing the unencapsulated thyroid follicles, were either homogenized in 0.9% saline solution or dissolved in normal sodium hydroxide. Aliquots of the solution were then measured for radioactivity by Geiger-Müller tube counting. For analysis of the metabolic forms of I^{131} in the thyroid, lower jaws of fishes killed at any given time interval were pooled and homogenized in physiological saline solution in a glass macerator. The saline supernatant of this extraction was digested at pH 8.5 with trypsin for 48 hours and chromatography of this hydrolysate was done according to the technique described by Lissitzky (1952). For identification of the unknown iodine compounds two different chromatographic solvents were used in each determination: n-butanol-acetic-acid and n-butanol-ammonia.

RESULTS

Quantitative

In respect to the thyroidal accumulation of the injected doses of I^{131}, three types of curves were obtained (Fig. 1). Uniformly low uptakes of approximately 10% were obtained in the saltwater form *F. majalis* either in salt- or relatively fresh water (survival was poor in fresh water) and in the brackish form *F. heteroclitus* in salt water (high tide). *F. heteroclitus* in relatively fresh water accumulated almost 30% of the injected I^{131} in its thyroid by 24 hours after injection, but lost it gradually over the next 5 days. In the freshwater form *F. diaphanus* it seems remarkable that the thyroidal accumulation of I^{131}, although plateaued at 22%, was still rising

* Iodine determinations were made by the Albert L. Chaney Laboratory, Glendale, California.

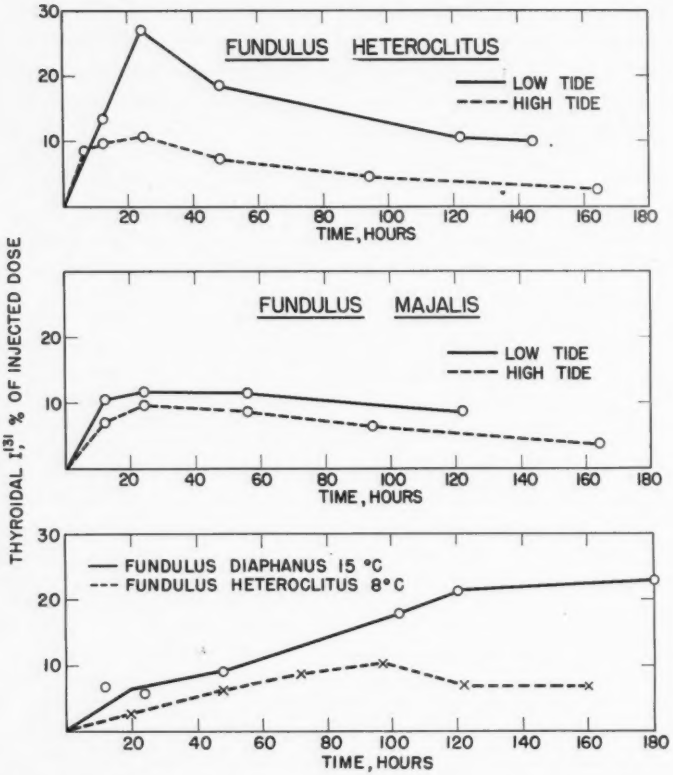

FIG. 1. Thyroidal radioiodine accumulation and loss over the period of a week in three species of *Fundulus*. Top: *Fundulus heteroclitus*, a brackish-water species capable of prolonged survival in both salt and fresh water. These animals were maintained at 22° C. in high tide (salt) and low tide (fresh) water taken from the mouth of the Wading River, Long Island, New York. Center: *Fundulus majalis*, a salt water species with poor survival in fresh water, maintained as above. Bottom: *Fundulus heteroclitus* maintained in high tide water (salt) from Wading River, and *F. diaphanus* maintained in running well water at the University of Michigan Biological Station. Water temperature was 15° C. or below. At these low temperatures peak accumulation of I^{131} is very slow; compare with top graph.

slightly after 7 days (Fig. 1). The two higher uptake curves differ markedly, therefore, in their time course.

Since the water temperature for the *F. diaphanus* was approximately 15° C., and since this factor could possibly affect the thyroidal behavior toward I^{131}, one experiment was carried out with *F. heteroclitus* kept at approximately 8° C. As may be noted in Figure 1, the total thyroidal up-

take under these conditions was reduced, from the value seen at 22° C., and a slowness to reach the peak I^{131} uptake was observable.

Qualitative

The iodine compounds, their order of appearance in the chromatograms, and their relative proportions did not differ in any significant way from the thyroxinogenic cycle of mammals, nor was there any appreciable differ-

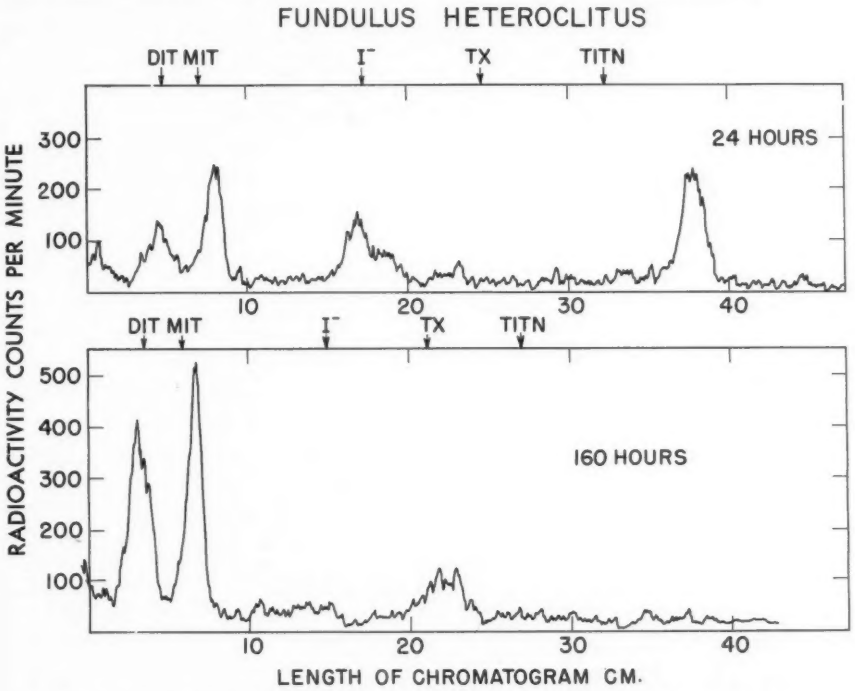

FIG. 2. Graphs showing distribution of radioactivity along radiochromatograms of thyroidal hydrolysates of *F. heteroclitus* kept in sea water for either 24 or 160 hours after injection of a tracer dose of radioiodine. Note the decrease in iodide and increase in thyroxine with time. MIT—monoiodotyrosine; DIT—diiodotyrosine; Tx—thyroxine; TITn—triiodothyronine; I—iodide.

ence between *F. diaphanus* and *F. heteroclitus* in their different types of aquatic environments (Figs. 2, 3). *F. majalis* was not examined chromatographically. In the earlier time intervals iodide and monoidotyrosine were present in larger proportions. The proportions of these substances decreased as diiodotyrosine and thyroxine increased at their expense. No triiodothyronine was detectable. An unknown I^{131} containing substance was found in the chromatograms of *F. heteroclitus* maintained in sea water.

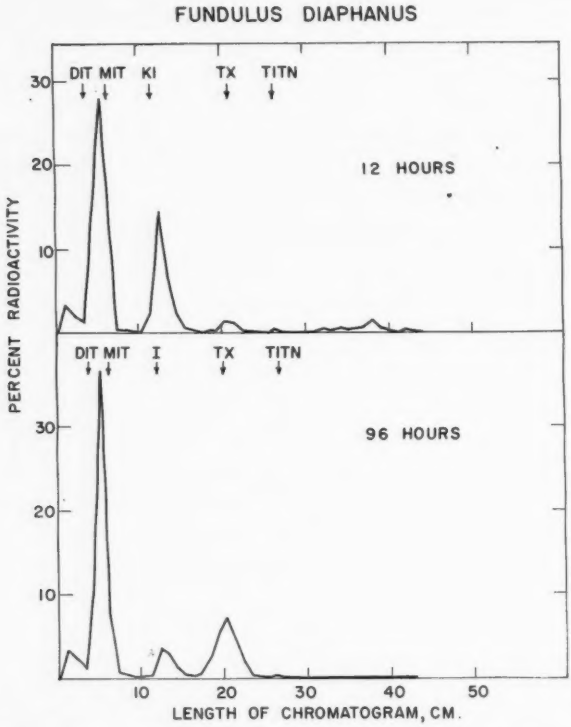

FIG. 3. Graphs showing distribution of radioactivity on radiochromatograms of thyroidal hydrolysates of *Fundulus diaphanus* kept in running well water. At 96 hours, this species shows the largest amount of thyroxine of any teleost species so far tested. MIT—monoiodotyrosine; DIT—diiodotyrosine; Tx—thyroxine; TITn—triiodothyronine; I—iodide.

This substance had an Rf value close to that of triiodothyronine, but it was easily distinguishable from this compound, as well as from any of the other known iodine compounds available to us, including diiodothyronine.

DISCUSSION

In respect to the iodine compounds formed and the order of their appearance, thyroid function in the three species of *Fundulus* we have studied resembles that of higher vertebrates. The presence of an unknown iodine-containing compound with a relatively high Rf value is possibly significant, but difficult to evaluate until the compound can be identified. The unknown substance was found only in the chromatograms of thyroid tissue of *F. heteroclitus* maintained in sea water. It decreased progressively in proportions at later time intervals.

On a quantitative basis, three kinds of iodine accumulation were found: 1) a low thyroidal uptake; 2) a high thyroidal uptake relatively quickly attained, and 3) a high thyroidal uptake very slowly attained. The low thyroidal uptake of tracer iodine occurred when either *F. majalis* or *F. heteroclitus* were kept in sea water. This could be due to the higher iodine content of the sea water, but there is, as yet, no basis on which we could eliminate other possibly influential constituents of sea water including the halogens: bromide fluoride and chloride. There is experimental evidence which indicates that in mammals both bromide and chloride influence thyroidal function (Axelrad and Leblond, 1952; Baumann and Mezger, 1949, and Sharpless and Anthony, 1943). Furthermore, because several workers have claimed that the thyroid gland of fishes has an osmoregulatory action (Koch and Heuts, 1942), it is still impossible to evaluate the role of environmental salinity alone as to its influence on thyroidal function. We are investigating several of these possible regulatory factors at the present time.

It should be noted that the response of thyroidal function in *F. majalis* to a fresher water ("low tide") environment was not the same as in *F. heteroclitus*. *F. majalis* lives almost exclusively in water of high salinity, the possible relation of this fact to the observed modest change in thyroidal iodine accumulation in response to lowered salinity is difficult to evaluate. Olivereau (1948) also found that thyroidal stimulation followed removal of two teleost species *Murena helena* and *Labrus bergylta* from sea water to fresh water. If the large I^{131} uptake of *F. heteroclitus* and the small one of *F. majalis* resulted from alteration of the same environmental factor, then perhaps, we are dealing with basic differences in endocrine balances and sensitivity of the two species.

Although the thyroids of *F. heteroclitus* and *F. diaphanus* in fresh water accumulated large fractions of an administered dose of I^{131}, they attained the peak level of accumulation at widely different times. If the *F. heteroclitus* were kept at a temperature approximately as low as that of the running well water used for *F. diaphanus*, their I^{131}-accumulation also was slowed down and reached a peak value very slowly. This would indicate that the rate of thyroidal radioiodine accumulation in *Fundulus*, as well as the rate of reaching the peak thyroidal value, tends to be a function of the environmental temperature. It was not possible to maintain the *F. diaphanus* at temperatures appreciably higher than 15° C. If the same temperature correlation could be expected for *F. diaphanus* and for *F. heteroclitus*, then the thyroidal I^{131} of this species should be considerably higher in fresh water at temperatures in excess of 15° C.

It is of special interest to note in Figure 1 that the thyroidal I^{131} in *F. diaphanus* continued to rise for more than 7 days, and even when the

experiment was concluded, it still was rising. Since the fish were being kept in continuously running water, this tracer iodine could not have been reabsorbed from the water, but must have come from depots in the peripheral tissues. So far as the authors are aware it has never been shown in higher vertebrates that the peripheral tissues could retain iodine in a storage form so that it could be fed into the circulation and the thyroid for periods as long as a week or more.

SUMMARY

Groups of *Fundulus majalis*, a salt-water species; *Fundulus heteroclitus*, a brackish-water species; and *Fundulus diaphanus*, a fresh-water species were injected with tracer doses of radioiodine Their thyroid tissue was analyzed by quantitative and chromatographic techniques at various time intervals after injection, up to one week.

The organic iodine compounds found were monoiodotyrosine, diiodotyrosine, thyroxine. One unknown compound which was not di- or triiodothyronine also was noted in *F. heteroclitus*.

When *F. majalis* or *F. heteroclitus* were kept in sea water their thyroidal I^{131} peak accumulation was about 10%. When they were kept in relatively fresh water after injection their peak thyroidal iodine accumulation was increased. The increase was slight in *F. majalis*, about 25%; it was very marked, almost threefold, in *F. heteroclitus*.

F. diaphanus kept in running water at 15° C. had a relatively high I^{131} thyroid uptake but within a week had not yet reached a peak level. *F. heteroclitus* kept at 8° C. had a lower I^{131} uptake in the thyroid, and reached the peak value very slowly.

The possible factors affecting these different patterns of thyroidal function are discussed.

REFERENCES

Axelrad, A. A. and C. P. Leblond: *Canad. Med. Assn. Jour.* **67**: 675–676. 1952.

Baumann, E. J. and N. Mezger: *Proc. Soc. Exp. Biol. & Med.* **70**: 536–540. 1949.

Harms, J. W.: *Ztschr. wiss. Zool.* **133**: 211–397. 1929.

Hoar, W. S., V. S. Black and E. C. Black: Some aspects of the physiology of fish. Publ. Ontario Fisheries Res. Lab., No. LXXI, University of Toronto Press. 1951.

Koch, H. J. and M. J. Heuts: *Ann Soc. Roy. Zool. Belgique.* **73**: 165–172. 1942.

LaRoche, G. and C. P. Leblond: *Endocrinology.* **51**: 524–545. 1952.

Lissitzky, S.: Biochimie des acides aminés iodés marqués par I^{131} dan le corps thyroide. Thesis, Université de Paris Paris, A.I.A. 1952.

Olivereau, M.: *C.r. Soc. Biol.* **142**: 176–177. 1948.

Robertson, O. H.: *Physiol. Zool.* **21**: 282–295. 1948.

Sharpless, G. R. and E. K. Anthony: *J. Nutrition.* **25**: 239–243. 1943.

Smith, D. C., S. A. Matthews: *Amer. J. Physiol.* **153**: 215–221. 1948.

DETERMINATION OF THE OXYGEN CONSUMPTION OF GROUPS OF RATS

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THE metabolic rate of small laboratory animals may be determined by either open or closed circuit respirometers. With the open circuit method, pure atmospheric air is inspired by the animal, then collected and analyzed for either oxygen or carbon dioxide concentration, or both. The composition of the inspired air must also either be known or determined. By difference, the rate of oxygen consumption or carbon dioxide production by the animal can be calculated for the period of observation. This method requires a good source of pure atmospheric air which is often difficult to obtain. A further drawback is the use of certain analytical procedures which are not necessary with the closed circuit system.

The closed circuit arrangement has the advantage of permitting: 1) the use of pure oxygen, and 2) the direct measurement of the volume of oxygen consumed by the animal. There are many designs of apparatus of this type (Reineke and Turner, 1950). Such units are usually limited to a capacity of only one or two rats so that experimental results are often open to question. These discrepancies have their origin in the variabilities observed from time to time in individual animals, and between any two animals. MacLagan and Sheahan (1949–50) express their views as follows: "It has been generally experienced that with single animals quantitative assays have not usually been possible on account of statistical variations." Similarly, Somers (1950) states that "the chief difficulty with biological assays [of thyroidal agents] is the extent of variation in the response of the individual animals, which necessitates the employment of relatively large numbers before an adequate degree of precision can be attained."

It is practically impossible to account for the different reactions of various animals to handling, environment, type of treatment, etc., and the different levels of metabolism which may exist at various times within a

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single animal. We have overcome many of these difficulties, however, by: 1) including a control, untreated animal group in each experiment; 2) using 5 or 6 animals in each group; 3) increasing to 6 the number of animals observed simultaneously under identical experimental conditions; 4) observing simultaneously representative animals from each of the control and treatment groups; and 5) determining individually the rate of oxygen consumption for each animal.

This is the method which was used in studies reported recently by two of us regarding the comparative potencies of L-thyroxine and L-triiodothyronine (Heming and Holtkamp, 1953a, 1953b) and the blocking by 'Dibenzyline'[3] of thyroxine-stimulated and epinephrine-stimulated increased oxygen consumption (Holtkamp and Heming, 1953a, 1953b). Since reporting these studies, there have been many inquiries regarding the details of the method, and the availability of the apparatus, or details of its construction. We hope that this detailed description of the unit in use at Smith, Kline and French Laboratories will answer the queries of those evincing an interest in its design and operation.

MATERIALS AND METHODS

Description of apparatus. The apparatus used for measuring the oxygen consumption of rats is shown in Figure 1. Further details of construction have been supplied in Figures 2–5. The apparatus consists essentially of a closed circuit respirometer. It is arranged in a constant temperature water bath (A) with two separate, unconnected compartments. The entire bath is supported on a level surface. The bath itself is made of 26-gauge stainless steel sheet. Six calibrated oxygen reservoirs, or bellows, (B) are suspended over distilled water in one compartment, while the animal chambers (C) are immersed in water in the other.

The water in the bath is maintained at 28° ± 0.2° C. by means of a thermoregulator (D) (Fenwal Expanding Shell Type, Arthur H. Thomas, Cat. No. 9646-A), a heater (E) (Immersion Tubular Type, 150-watt, Arthur H. Thomas, Cat. No. 6147-B) and a circulating pump (F) (Arthur H. Thomas, Cat. No. 8490, or 8496—the latter is preferred). Power is supplied to these three units by way of the electrical control box (G) having a 110-volt input (H), the pilot light (I), and output wires (J) and (K) which lead to the thermoregulator and circulating pump, respectively. A third wire, not shown, provides a connection directly with the heater. For convenience, a clock time-switch (Arthur H. Thomas, Cat. No. 3655-S) in the 110-volt input circuit permits automatic preliminary heating of the bath prior to use. The thermometer (L) is incorporated as a check on the temperature.

The tank outlet (M), leading to the circulating pump, has an opening on the side of the pipe (Fig. 2) rather than on the end so that if an animal chamber (C) is placed close to it, the opening will not be blocked, thereby interfering with circulation of the water. To assure more precise temperature regulation, the tank inlet (N) is located beneath the thermoregulator thus permitting the water to circulate from the thermoregulator toward the heater. In this compartment the depth of water is adjusted daily so that there is from 1/2″ to 3/4″ of water above the animal chambers when all are in position.

[3] Phenoxyisopropyl-N-benzyl-β-chloroethylamine hydrochloride.

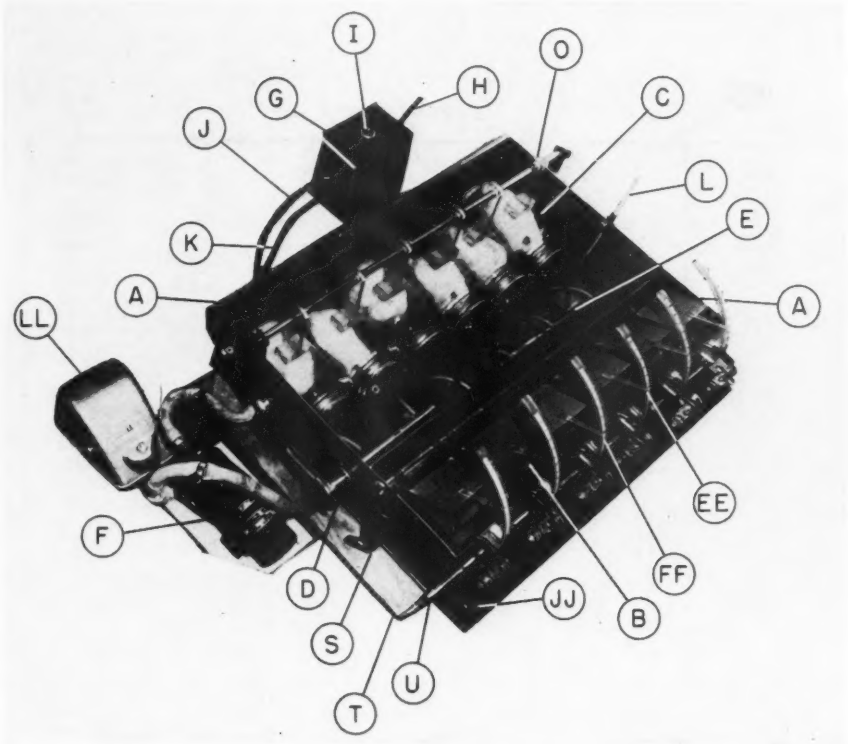

FIG. 1. Apparatus used for measuring oxygen consumption of rats.

The animal chambers (C) consist of square-type, quart Mason jars (Atlas, "R") having a screw-top with separate jar lids and jar rings. A hole is bored through the jar lid to which is soldered a 1/4″ O.D. copper tube. This tube is connected, by means of rubber tubing, to the oxygen supply tube from the bellows. Passage of oxygen through this tube is controlled by means of a Mohr pinchcock which provides a means of (1) flushing and filling the bellows with oxygen *prior to* attachment of the animal chamber, and (2) making oxygen readily available to the chamber after attachment. A rubber washer or gasket is inserted between the jar ring and jar lid to provide a tight seal.

The jars, or animal chambers, are kept submerged in the water bath by the movable plastic posts (0) above each jar. Each post is made of 3 pieces of "Plexiglas" and consists of a lower block, a bar, and a short piece of firm-wall tubing (Fig. 3). The use of the plastic tubing permits the post to swing freely on the upper bar support of 3/8″ stainless steel rod (P). It also permits the post to be moved along the length of the bar as desired. At each end the steel bar is supported by a metal support (Q) which is notched at its upper end. This allows the bar (P) to be adjusted up or down by merely loosening the wing nut (R).

Each animal chamber is provided with a non-removable screen platform (8-mesh wire) measuring about 5″×2-1/2″. Separate wire (8-mesh) supports, spaced at a distance

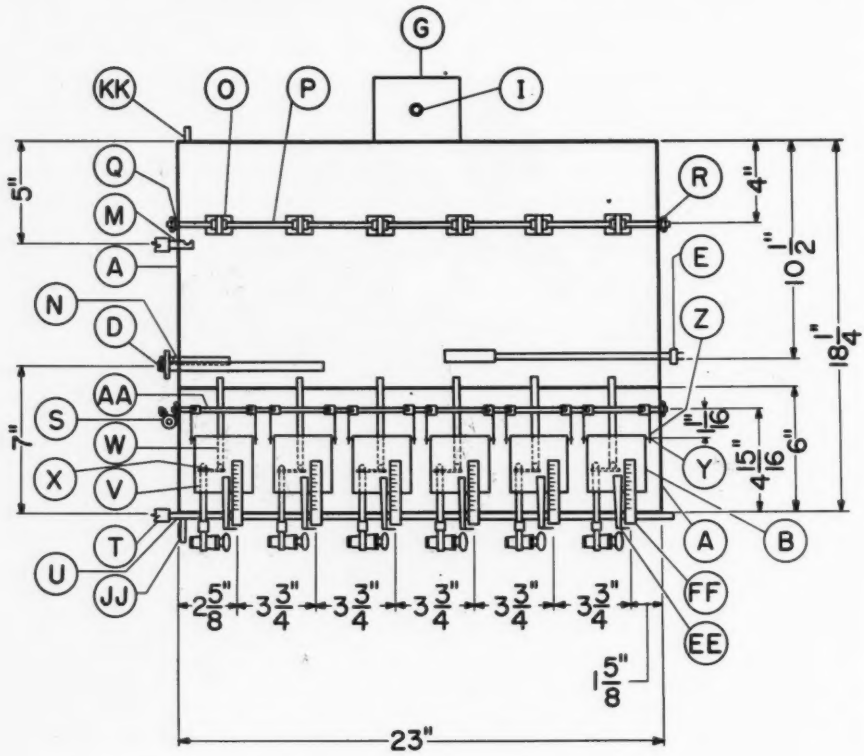

FIG. 2. Schematic top view of apparatus.

of 1-1/4″, are soldered to the platform for its entire length. These supports maintain the platform 1/2″ above the floor of the jar. In the space between these supports may be placed a removable envelope or tube (about 1-1/8″×4-3/4″×1/2″) made of 40-mesh stainless steel wire containing the soda and lime for absorbing the expired carbon dioxide.

The front compartment of the bath houses the bellows (B) which are supported on water maintained at constant depth (5-1/8″) by means of a water level regulator (S), (Arthur H. Thomas, Cat. No. 9876 is satisfactory). Oxygen for use in the experiments is first transferred from a commercial tank to a four-liter bottle by the displacement of water. Subsequent water displacement of the oxygen in the bottle then provides the oxygen for the individual bellows (B) by way of 1) the oxygen inlet tubing (T); 2) the copper manifold (U) consisting of a 3/8″ O.D. main tube and 1/4″ O.D. branch outlets; 3) the individual stopcock ("Pyrex," S.T. No. 2, 8 mm.); and 4) the 1/4″ O.D. copper tubing inlet (V). The latter are connected by rubber tubing. The outlet tube (W) of the bellows transports the oxygen to the animal chamber. It, like the inlet tube, is made of 1/4″ O.D. copper tubing. The opening of both tubes within the bellows is 5/16″ above the surface of the water and is flared slightly to reduce the possibility of accidental entry of water. For strength the two tubes are joined below the surface of the water by a solid copper bar (X), 1/16″ in diameter. Furthermore, tube (W) is soldered to the partition

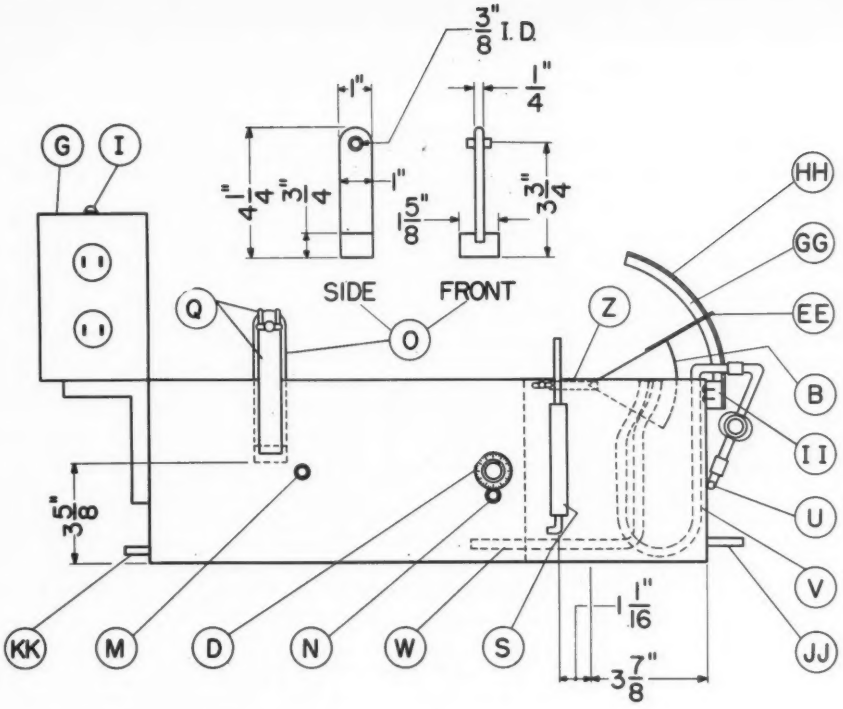

FIG. 3. Schematic side view of apparatus and detailed drawing of plastic posts (0) which keep animal chambers submerged in water.

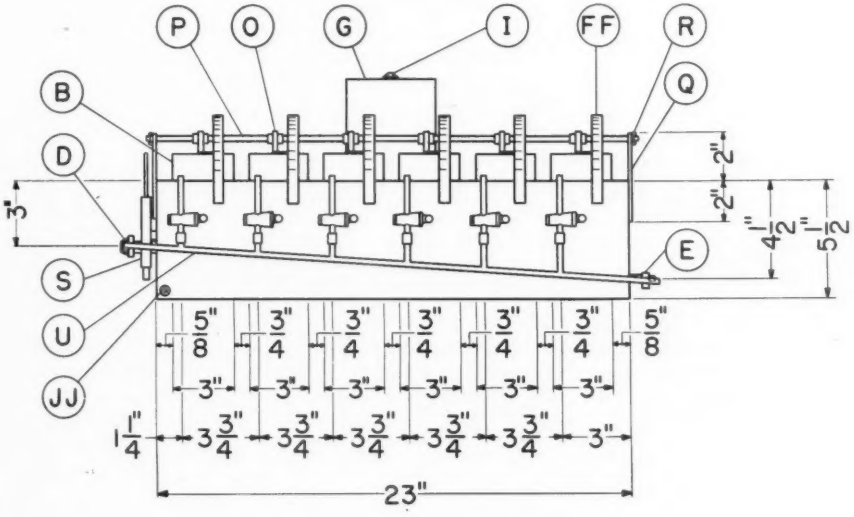

FIG. 4. Schematic front view of apparatus.

of the tank where it passes through the wall into the back compartment; tube (V) is clamped as well as soldered to the front wall at a point below its passage over the front wall of the tank.

The bellows (B) are made of 0.005″ shim-stock brass with the fulcrum, an essentially frictionless and oilless hinge at its back edge. A brass rod (Y), (1/16″ O.D. and 3-3/8″

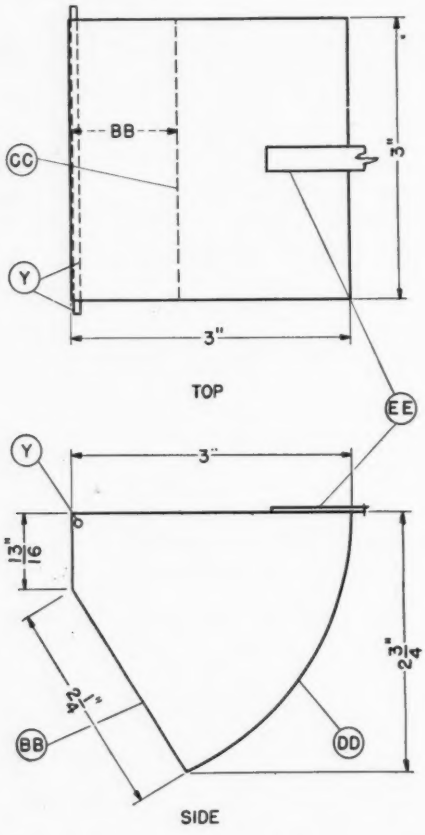

FIG. 5. Schematic diagram of bellows showing top and side views.

in length) is passed through the bellows cross-wise, leaving a 3/16″ bearing on each side. Each end of the rod where it passes through the wall of the bellows is well soldered to prevent leakage. These 3/16″-projections (bearings) are inserted in a hole so as to move freely in one end of the support (Z). The support (Z) is made by attaching brass tubing (1/4″ I.D. and 3/8″ long) to a 1/16″-thick brass plate (1-3/8″×about 5/16″, but somewhat narrower at the bellows end) which is bored with a 1/4″ hole to match the tubing, and the hole for the brass-rod fulcrum of the bellows. These brass supports (Z), two per bellows, are spaced properly and then anchored with "4-40 Allen" set screws onto the

stationary 1/4″ O.D. stainless steel rod (AA), which crosses the complete width of the tank.

Two views of the bellows (B) are shown in Figure 5. Observe that there is only one opening (BB) into the bellows and that this is on the under-surface. It is 2-1/4″×3″. The front edge (CC) of the opening is also the lower edge of the solid curved surface (DD) as seen in the side view. The pointer (EE) for the bellows consists of a plastic arm (2-3/4″ ×1/16″×about 1/4″—somewhat narrower on the free end). Attached to this is an 0.040″ wire 5/8″ in length. As the bellows rises and falls, the pointer moves up and down allowing the wire to pass along the front of the calibrated graduated scale (FF) engraved on the curved plastic bar. The latter is made by cementing together 3 pieces of plastic: 1) an arc (GG) cut by band saw from 1/8″ plastic and having an O.D. radius of 4-13/16″ and I.D. radius of 4-5/16″, 2) a 1/8″-thick piece of plastic (HH) having a width of 1/2″ which is bent and molded to fit the outer surface of the arc; and 3) the attachment block (II), (1-1/8″×3/4″×1/2″) to which both of the former are cemented. (Throughout the apparatus, "Plexiglas Cement" was used when attaching two pieces of plastic; however, "Duco Household Cement" was used to attach the plastic to metal.) This assembly is attached to the front of the tank by screws. The molded, or bent, piece of plastic (HH) bears the scale (FF) calibrated in 5 ml. divisions. The scale is engraved after calibration as described below.

In order to clean the bath conveniently, one drain outlet (JJ), is provided for the front compartment, and another (KK), for the back. In addition, as an adjunct to operation of the unit, a timer (LL) (Arthur H. Thomas Catalog No. 9315-M) is kept in easy view of the operator.

Calibration of bellows. By means of rubber tubing, a 10 ml. hypodermic syringe is attached to a 2-way stopcock, the second opening of which is attached to the bellows. The third opening of this stopcock is either open to the environment when calibrating with air, or attached to a "Y" tube when calibrating with oxygen. For the latter, one limb of the "Y" tube is attached to the oxygen supply (under slightly greater than atmospheric pressure) and the other limb is attached to a rubber tubing regulated by a pinch clamp. The latter is used as a release valve to assure environmental atmospheric pressure in the syringe prior to the transport of the oxygen from the syringe to the bellows. With the use of both air and oxygen, the individual bellows may be calibrated by first adding, and later withdrawing, 10 ml. aliquots of the gas to, or from, the bellows. The position of the pointer (EE) is marked on the scale (FF) with a fine pencil. After verification, these points are engraved on the plastic. We have been unable to detect any difference between the methods of addition and withdrawal. With care, on 3 successive trials on a single bellows, the 10 ml.-divisions are duplicable. The 5 ml.-division marks may be placed by interpolation.

Calculation. For our routine work, the volume of oxygen consumed is adjusted to standard temperature and pressure, and the final results are expressed as liters consumed per square meter surface area per hour (L./sq.M./hr.). This is determined from the following formula:

$$(1) \qquad \text{L./sq.M./hr.} = \frac{V}{t} \times \frac{P}{760} \times \frac{273}{T} \times \frac{10{,}000}{(\text{Wt.})^{2/3} \times 10} \times \frac{1}{1000} \times \frac{c}{10}$$

L./sq.M./hr. = volume of oxygen consumed per square meter surface area per hour corrected to standard temperature and pressure

V = mean observed volume (ml.) of oxygen consumed

t = time (hr.)

P = barometric pressure (mm.) corrected for vapor tension and per cent saturation at time of test

T = absolute temperature at time of test

Wt. = weight of animal (gm.)

$\frac{10{,}000}{(\text{Wt.})^{2/3} \times 10}$ = formula for converting body wt. (in gm.) to surface area (in sq.m.) (Hill and Hill, 1913)

$\frac{1}{1000}$ = factor converting cubic centimeters to liters

$\frac{c}{10}$ = factor correcting for error in syringe used to calibrate bellows.

In our laboratories, the temperature (T) and time (t) are kept constant at 28° C. and 0.25 hr., respectively. For calibration, c was equal to 10.2. Substitution in the above formula (1) yields the following:

$$(2) \qquad \text{L./sq.M./hr.} = \frac{V}{0.25} \times \frac{P}{760} \times \frac{273}{301} \times \frac{10{,}000}{(\text{Wt.})^{2/3} \times 10} \times \frac{1}{1000} \times \frac{10.2}{10}$$

which can in turn be simplified to:

$$(3) \qquad \text{L./sq.M./hr.} = \frac{V \times P}{(\text{Wt.})^{2/3}} \times 0.00487.$$

The following is a sample calculation: An intact control rat weighing 150 gm. had an observed rate of oxygen consumption of 56, 51 and 53 ml. (uncorrected) for the three respective consecutive 15-minute periods. The mean oxygen consumption per 15-minute period was, therefore, 53 ml. The corrected barometric pressure (P) for that day was 736.5 mm. Substituting in equation (3):

$$\text{L./sq.M./hr.} = \frac{53 \times 736.5}{(150)^{2/3}} \times 0.00487 = 6.8.$$

For ease of calculation, we use a table prepared in our laboratory which converts measured body weight (gm.) to the value of $(\text{Wt.})^{2/3}$.

Animal Studies. To determine the variability, or possible experimental error, involved, in the use of this apparatus, the rate of oxygen consumption of 5 intact control (Group 1), 6 thyroidectomized control (Group 2), and 5 treated, thyroidectomized (Group 3) male rats was determined at semi-weekly intervals for a series of nine tests. The treated rats received 24 μg. of L-thyroxine sodium pentahydrate/kg./day in alkaline saline solution by subcutaneous injection. The treatment started after the first and ended 24 hours before the ninth determination. Control rats received only the alkaline saline solution. All rats (Albino Farms) weighed between 125 and 175 gm. at the time of the surgical thyroidectomy. Thirty days elapsed between the time of these operative procedures and the first observations on rate of oxygen consumption. Thyroidectomized rats were maintained on 1% calcium gluconate as the drinking fluid. A 24-hour fast preceded each determination.

For each run, two animals from each of the three groups were placed in the chambers (C). A 30-minute acclimatization period was allowed before starting the test. Then, the oxygen consumption of each rat was measured for 3 successive 15-minute periods by

means of the pointer (EE) and the scale (FF). Occasionally, when an animal was unduly active during a 15-minute observation period it was recorded, but the value for that period was not considered in the final result.

In successive runs, the various groups were rotated through the respective chambers. Thus, chambers used for intact control rats (Group 1) for the first run, held control thyroidectomized rats (Group 2) for the second run and treated thyroidectomized rats (Group 3) during the third run. Such rotation may minimize any variability inherent in the apparatus.

An important part of this procedure as a whole is the fact that each experiment includes intact, untreated control and thyroxine-treated, control animals as well as the test animals.

As precautionary measures, the following two additional procedures were carried out on the morning of each day that the apparatus was used. First, each of the bellows was inverted, and air was blown through the tube (W), connecting the bellows to the animal chamber. This assured a free passageway for oxygen by removing water of either condensation or accidental entry. Second, each of the bellows was returned to normal position and flushed 3 to 5 times by the alternate inlet and outlet of oxygen through the use of the inlet stopcock and the pinch clamp near the animal chamber.

RESULTS AND DISCUSSION

The experimental results have been expressed in each of two different ways. One (Table 1) is an expression of the group response, the other (Table 2) of the individual animal response. A comparison shows that for each of the three respective animal groups (intact control, thyroidectom-

TABLE 1. MEAN ±S.D. OF OXYGEN CONSUMPTION SHOWING GROUP RESPONSE OF 5 CONTROL INTACT, 6 CONTROL THYROIDECTOMIZED, AND 5 TREATED THYROIDECTOMIZED RATS FOR EACH OF SUCCESSIVE SEMI-WEEKLY DETERMINATIONS

Determination No.	Oxygen consumption (L./sq.M./hr.)[1]		
	Control intact rats (Group 1)	Control thyroidectomized rats (Group 2)	Treated thyroidectomized rats[2] (Group 3)
1	6.5 ±0.2	5.0 ±0.6	—
2	5.9 ±0.2	4.9 ±0.6	—
3	5.7 ±0.2	4.8 ±0.3	—
4	6.2 ±0.9	4.7 ±0.4	—
5	5.8 ±0.2	4.8 ±0.4	—
6	6.0 ±0.8	4.7 ±0.6	—
7	6.3 ±0.7	4.8 ±0.5	6.0 ±0.5
8	6.0 ±0.7	4.7 ±0.7	6.2 ±0.3
9	6.3 ±1.0	4.9 ±0.7	6.2 ±0.6
Mean ±S.D. of above means	6.1 ±0.3	4.8 ±0.1	6.1 ±0.1
Mean ±S.D. of above S.D.'s	0.5 ±0.3	0.5 ±0.14	0.5 ±0.2

[1] Mean ±S.D.

[2] Treatment, beginning after 1st determination, with L-thyroxine sodium pentahydrate at a daily subcutaneous dose of 24 μg./kg. First 6 determinations made before plateauing of response and therefore not included in these data showing experimental error of method.

TABLE 2. MEAN ±S.D. OF OXYGEN CONSUMPTION SHOWING INDIVIDUAL RESPONSE OF EACH OF 5 CONTROL INTACT, AND 6 CONTROL THYROIDECTOMIZED RATS FOR 9 SUCCESSIVE SEMI-WEEKLY DETERMINATIONS. SIMILAR DATA ARE INCLUDED FOR EACH OF 5 TREATED THYROIDECTOMIZED RATS BUT FOR ONLY 3 SUCH PERIODS

	Control intact rats (Group 1)		Control thyroidectomized rats (Group 2)		Treated thyroidectomized rats[2] (Group 3)	
	Rat No.	Oxygen consumption (L./sq.M./hr.)[1]	Rat No.	Oxygen consumption (L./sq.M./hr.)	Rat No.	Oxygen consumption (L./sq.M./hr.)
	1	6.2±0.7	8	5.1±0.4	19	5.5±0.3
	2	5.8±0.8	9	4.2±0.9	21	6.2±0.2
	3	5.7±0.5	10	4.8±0.2	22	6.4±0.7
	6	6.7±0.8	27	4.4±0.4	40	6.3±0.4
	7	6.0±0.4	29	4.9±0.2	41	6.3±0.0
			31	5.5±0.3		
Mean ±S.D. of above means		6.1±0.4		4.8±0.5		6.1±0.4
Mean ±S.D. of above S.D.'s		0.6±0.2		0.4±0.3		0.3±0.3

[1,2] Same as for Table 1.

ized control, and treated thyroidectomized) the mean of the standard deviations is approximately 0.5 L./sq.M./hr. with a range of 0.3 to 0.6 L./sq. M. /hr. This standard deviation includes, of course, the total variability inherent in both the apparatus and the animals. As a further measure of the precision of the method, if a calculation is made in terms of the group response (Table 1), the mean of the 21 standard deviations (0.5 L./sq.M./hr.) is only 9% of the mean of the 21 means (5.6 L./sq.M./hr.). Also, if a similar calculation is made on the data expressed in terms of individual response (Table 2), the mean of the standard deviations (0.45 L./sq.M./hr.) is 8% of the mean of the means (5.6 L./sq.M./hr.).

Plus or minus two standard deviations include 95.46% of the individual measurements (Youden, 1951). Such limits are often used either as an estimate of the error of a single measurement (Berkson, Magath and Hurn, 1943) or as the confidence limits at the 5% level, within which the average would lie if based on a large number of measurements. However, for our purposes, we have corrected for small samples (Youden, 1951) and calculated from the above standard deviations, that the estimate of error of a single measurement is ±17 to 19% for this method. For comparison, this value is approximately the same as the ±16% possible error of a single determination of the erythrocyte count, utilizing the usual pipette and chamber count method (Berkson, Magath, and Hurn, 1943). In view of the long acceptance of this method for determining the erythrocyte count, we believe our determination of the rate of oxygen consumption, is quite

accurate and very useful in the determinations of calorigenic potency of various compounds.

In considering whether to use intact or thyroidectomized rats, it can be seen (Table 1) that the mean oxygen consumption of the control intact rats is somewhat more variable between observations (S.D. of mean of means $=0.3$ L./sq.M./hr.) than that of either of the thyroidectomized groups (S.D. of mean of means $=0.1$ L./sq.M./hr.). On the other hand, the standard deviation per observation, is approximately the same in each of the 3 respective groups as evidenced by the mean of the standard deviations (0.5 L./sq.M./hr.) for each group. This would suggest that 1) the variability within a single observation is approximately the same in intact rats as in thyroidectomized rats, but 2) the variability between one observation and another appears to be greater for intact than for thyroidectomized rats. The inherent error of the method would, therefore, probably be lower if thyroidectomized rats were used for chronic experiments involving multiple observations, but for single observation experiments, the use of intact rats would be satisfactory. For routine work in our laboratories, we always include an untreated control group of rats in each experiment whose oxygen consumption on the day of the test is used as the daily reference point for all comparisons in that particular experiment.

For final emphasis, we ascribe the accuracy of this method to the following factors: 1) similarity in weight, age, and sex of the rats in the control and treatment groups; 2) use of 5 or more animals per group; 3) simultaneous observation of representative rats (6 rats in our unit, but its capacity could be enlarged); and 4) precision of the apparatus itself.

SUMMARY

A convenient and reliable method is described (including detailed drawings of the apparatus) for determining simultaneously the individual oxygen consumption of a group of rats. Its reliability approximates the accuracy of the long accepted erythrocyte count determination. In actual use with intact control, thyrodectomized control, and treated thyroidectomized rats, the estimate of error of a single determination (i.e., the confidence limits at the 5% level, within which the average would lie if based on a large number of measurements) is $\pm 18\%$ of the mean.

Acknowledgment

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REFERENCES

BERKSON, J., T. B. MAGATH AND M. HURN: cited by J. C. Todd and A. H. Sanford, Clinical Diagnosis by Laboratory Methods, 10th Edition, W. B. Saunders Company, Philadelphia, 1943, pp. 226–227.

HEMING, A. E. AND D. E. HOLTKAMP: *J. Clin. Endocrinol. & Metab.* **13**: 880. 1953a.

HEMING, A. E. AND D. E. HOLTKAMP: *Fed. Proc.* **12**: 330. 1953b.

HILL, A. V. AND A. M. HILL: *J. Physiol.* **46**: 81. 1913.

HOLTKAMP, D. E. AND A. E. HEMING: *J. Clin. Endocrinol. & Metab.* **13**: 853. 1953a.

HOLTKAMP, D. E. AND A. E. HEMING: *Fed. Proc.* **12**: 331. 1953b.

MACLAGAN, N. F. AND M. M. SHEAHAN: *J. of Endocrinol.* **6**: 456. 1949–50.

REINEKE, E. P. AND C. W. TURNER: in C. W. Emmens, Hormone Assay, Academic Press, Inc., Publishers, New York, 1950, p. 493.

SOMERS, G. F.: *The Analyst* **75**: 537 (quoted from p. 540). 1950.

YOUDEN, W. J.: Statistical Methods for Chemists, John Wiley and Sons, Inc., New York, 1951, pp. 12, 18–20.

ADRENOCORTICAL AND THYROID FUNCTION IN THE CASTRATE MALE DOG[1]

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INTRODUCTION

THE extensive and somewhat conflicting literature on the effect of gonadectomy on the thyroid and adrenal has been reviewed by a number of authors (Andersen and Kennedy, 1933; Smith, 1946; Blumenfeld, 1939; Farbman, 1944). Most of the older studies, however, have been limited to observations on the weight and histology of these organs, and there is relatively little data on thyroid and adrenal function. Nowell's observation (1953) that the gonadectomized rat has a decreased ascorbic acid fall in response to cold stress emphasizes the question of whether or not the pituitary-adrenal axis is capable of responding normally to stress in the castrate animal. The present study was therefore undertaken to determine the effect of castration on adrenocortical and thyroid function in the male dog.

MATERIALS AND METHODS

Eight adult mongrel dogs weighing 9.6 to 11.5 kg. were castrated. Two weeks later their eosinopenic response to the intramuscular administration of a single dose of 40 international units of ACTH was tested. Three days later, they were subjected to a standard surgical trauma ("standard operation"), and then, two days later, to forced immobilization, and their eosinopenic response to these stresses determined.

Three weeks after castration, the dogs were given intravenously a tracer dose of radioactive iodine and the iodine uptake of their thyroid glands determined by a standard *in vivo* counting technique. Details of this method will be reported elsewhere. The animals were counted on a special scintillation counter 24, 48 and 72 hours after injection of the tracer. By counting an indifferent as well as the thyroid area, and making an appropriate correction, the per cent of the injected dose in the thyroid gland was calculated for each dog at each determination.

One month after castration, the left adrenal was surgically removed and 14 days later the right adrenal was similarly removed. Three additional castrate dogs were given radioactive iodine uptakes six weeks after castration, but were not included in the rest of the experiment.

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The standard surgical trauma consisted of incising the lateral aspect of the flank for a distance of 6 cm. under pentothal induced ether anesthesia. The underlying muscle was then split to expose the underlying structures, bleeding was controlled, and the wound closed in layers. The procedure was timed to take exactly ½ hour.

Forced immobilization was performed by placing the dog in a special canvass sling which prevents motion for a period of two hours.

Eosinophil counts were drawn in balanced oxalate, kept in the refrigerator, and counted within 24 hours by a modification of the method of Dunger (Recant, Hume, Forsham and Thorn, 1950). Eosinophils were counted before and 4 hours after ACTH administration and immobilization and before and 6 hours after standard surgical trauma. The per cent eosinophil fall for the respective periods was calculated and the results analyzed statistically.

The removed adrenals were washed once in saline, dissected free of connective tissue and fat, blotted once on gauze, and weighed immediately on a laboratory balance. They were then fixed in 10% neutral formalin for at least two weeks. Frozen sections were stained with sudan black B, sudan IV, and the Schiff reagent. In addition, frozen sections were examined under the polarizing microscope before and after acetone extraction. Finally, permanent paraffin sections were stained with hematoxylin and eosin and by the technique of Klüver and Barrera (1953).

Adrenal weights were expressed in mg./kg. of dog body weight. The ratio of the right to the left adrenal weight was expressed as a per cent by the following formula:

$$\left[\frac{\text{right adrenal weight}}{\text{left adrenal weight}} - 1.00\right] \times 100 = \text{the per cent variation in weight.}$$

Dogs were fed a standard diet of *Ken-L-Biskit*,[3] and all animals were in good health throughout the experiment.

Normal control dogs were handled in a similar manner. Forty normal dogs were subjected to standard operation and to immobilization and their eosinopenic responses analyzed. Radioactive iodine uptakes were determined on 25 normal dogs. Six normal dogs were subjected to left adrenalectomy and two weeks later to right adrenalectomy to determine the extent of compensatory hypertrophy occurring. In addition, two normal dogs were subjected to repeated trauma designed to be equivalent to that to which the experimental animals were subjected before adrenalectomy, and these dogs then had left, followed in two weeks by right, adrenalectomy. The data on the normal animals subjected to unilateral adrenalectomy has been published as part of another study (Ganong and Hume, 1954).

Finally, radioactive iodine uptakes were performed on six normal dogs who first were subjected to trauma comparable to that received by the castrate animals.

RESULTS

In normal dogs, 40 international units of ACTH injected intramuscularly produced an eosinopenia of $61 \pm 6.4\%$[4] in 4 hours. The castrate dogs in the present series showed a $75 \pm 3.5\%$ fall. Similarly, immobilization and standard surgical trauma produced in the normal dog a $66 \pm 2.1\%$ and a $78 \pm 1.9\%$ eosinopenia respectively. In the castrate dogs, immobilization produced a $75 \pm 5.6\%$ fall and standard surgical trauma, a $69 \pm 5.0\%$ fall.

[3] Quaker Oats Co., Chicago, U.S.A.

[4] Standard error of the mean.

None of these results in the castrate animals is significantly different from the normal.

The left adrenals removed one month after castration had a mean weight of 52.8 ± 2.4 mg./kg., and histologically showed evidence of mild hypertrophy. The normal, unstressed dogs had a mean left adrenal weight of 42.0 ± 2.4 mg./kg., but in the two animals subjected to trauma comparable to that of the castrate dogs before adrenalectomy the mean weight was 54.8 mg./kg. The right adrenal glands of the castrate dogs removed two weeks later weighed 67.9 ± 3.1 mg./kg. and showed definite hypertrophic changes. The right adrenals were $28.8 \pm 2.7\%$ larger than the left. This weight increase is slightly larger than the compensatory hypertrophy seen in the normal dogs ($20 \pm 4.6\%$), but the difference is not statistically significant.

The castrate dogs had $11.3 \pm 1.5\%$ of the injected radioactive iodine in the thyroid gland at 24 hours, while $14.5 \pm 2.2\%$ was present at 48, and $18.0 \pm 2.1\%$ was present at 72 hours. The unstressed normal animals, on the other hand, took up 19.5 ± 1.9, 28.7 ± 3.0, and $32.0 \pm 3.0\%$ of the injected dose at 24, 48, and 72 hours. The difference between these values is statistically significant at 48 and 72 hours. ($P < 0.02$) Normal animals previously subjected to trauma comparable to that which the castrate animals received took up 12.1 ± 1.2, 19.5 ± 1.7, and $25.0 \pm 1.0\%$ of the injected dose, and these figures are not significantly lower than those of the normal animals.

DISCUSSION

Szego and White (1951) as well as Nowell (1953) suggested that the adrenocortical response to stress was abnormal in the castrate animal. On the other hand, Zeckwer (1945) found normal responsiveness in the rat. It is apparent from the present data that there is no significant difference between the eosinopenic response to stress in the castrate dog and the normal dog. Use of the eosinophil as a measure of adrenocortical function has been justifiably criticized (Thorn, 1952). However, in the dog both the standard surgical trauma and forced immobilization produce a reproducible eosinopenia which can be regularly abolished by hypophysectomy and adrenalectomy, but not by adrenal demedullectomy. (Ganong and Hume, unpublished.) In addition, recent data from this laboratory shows that peripheral blood corticoid levels rise markedly with both surgical and immobilization stress in the dog (Steenburg and Ganong, unpublished). For these reasons, it seems justifiable to use the eosinophil as a measure of adrenocortical function for these two particular procedures in this particular species.

The literature on adrenal size changes following gonadectomy is confusing (Smith, 1946; Andersen and Kennedy, 1933). The left adrenals of cas-

trate dogs, surgically removed one month after gonadectomy, are slightly heavier than those of the unstressed normal animal and histologically, they show mild hypertrophic changes. However, it must be remembered that these animals were subjected to a number of stressful procedures before adrenalectomy, and their adrenals must therefore be compared to those of normal animals subjected to comparable degrees of stress. When such a comparison is made, there is no difference between the castrate and the normal male dog.

In addition, the compensatory hypertrophy response, which depends on an intact pituitary (MacKay and MacKay, 1924) is normal in these castrate dogs. There is thus no evidence for depressed adrenocortical function either at rest or in response to stress in the castrate dog.

In most species, the literature is in general agreement that castration depresses the thyroid (Andersen and Kennedy, 1933; Maqsood, 1950; Betz, 1952), although McGavack (1951) feels that this depression may be temporary. On the other hand, Paschkis, Cantarow and Peacock (1950) found no difference in radioactive iodine uptake between the normal and the castrate female rat. It is of interest, therefore, that in the present study, the iodine uptake is slightly, but definitely, depressed three weeks after castration in the male dog. Wase and Repplinger (1953) have reported that stress lowers the iodine uptake, but the degree of trauma involved in these studies does not depress the uptake of the normal dog significantly. In addition to the present data, other unpublished studies from this laboratory show that in the dog, surgical stress has no effect on the biologic half-life of iodine in the thyroid gland. The lowered uptake in castrate dogs cannot be explained, therefore, on the basis of trauma alone, and probably represents a physiologically significant finding.

SUMMARY

1. Adrenocortical function has been evaluated in eight castrate male dogs by testing their eosinopenic response to ACTH administration, a standard surgical trauma, and forced immobilization. The histologic response of their adrenals to surgical trauma as well as the changes in gland weight were also observed and the degree of compensatory hypertrophy of the remaining adrenal after unilateral adrenalectomy determined.

2. Thyroid function was evaluated by determining the per cent of an injected dose of radioactive iodine taken up by the thyroid gland *in vivo* at 24, 48 and 72 hours.

3. The eosinopenic response to ACTH, surgical trauma and immobilization was normal in these animals. Adrenal weight was slightly increased by repeated stress and there was mild histologic hypertrophy, similar to that

seen in the normal dog subjected to similar procedures. A normal 28 per cent compensatory hypertrophy occurred.

4. Thyroid iodine uptakes were significantly lower than those observed in normal dogs.

5. Repeated trauma in normal dogs did not lower thyroid iodine uptake significantly.

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REFERENCES

ANDERSEN, D. H. AND H. S. KENNEDY: *J. Physiol.* **79:** 1, 1933.
BETZ, H.: *C. rend. Soc. biol.* **146:** 325, 1952.
BLUMENFELD, C. M.: *Endocrinology* **24:** 723, 1937.
FARBMAN, A. A.: *J. Clin. Endocrinol.* **4:** 17, 1944.
GANONG, W. F. AND D. M. HUME: *Endocrinology* **55:** 474. 1954.
KLÜVER, H. AND E. BARRERA: *J. Neuropath. & Exper. Neurol.* **12:** 400, 1953.
MACKAY, E. M. AND L. L. MACKAY: *J. Exper. Med.* **43:** 395, 1926.
MAQSOOD, M.: *Nature* **166:** 735, 1950.
MCGAVACK, T. H.: *The Thyroid*, p. 144, C. V. Mosby Co., St. Louis, 1951.
NOWELL, N. W.: *J. Endocrinol.* **9:** XVII, 1953.
PASCHKIS, K. E., A. CANTAROW AND W. C. PEACOCK: *Proc. Soc. Exper. Biol. & Med.* **68:** 485, 1948.
RECANT, L., D. M. HUME, P. H. FORSHAM AND G. W. THORN: *J. Clin. Endocrinol.* **10:** 187. 1950.
SMITH, D. E.: *Am. J. Physiol.* **146:** 133. 1946.
SZEGO, C. M. AND A. WHITE: *Endocrinology* **48:** 576, 1951.
THORN, G. W.: *Am. J. Med.* **14:** 139, 1953.
WASE, A. W. AND E. REPPLINGER: *Endocrinology* **53:** 451, 1953.
ZECKWER, I. T.: *Proc. Soc. Exper. Biol. & Med.* **60:** 66, 1945.

THE HYDROLYSIS OF RAT THYROGLOBULIN BY THYROIDAL ENZYMES[1]

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THE presence of proteolytic substances in the thyroid gland was first demonstrated by the digestion of a gelatin film with follicular colloid, obtained by microdissection from rat thyroids (De Robertis, 1941). Later, whole thyroid extracts were shown to exhibit hydrolytic activity at acid pH against edestin (Dziemian, 1943), and hemoglobin (Kamner *et al.*, 1950; Weiss, 1953). The proteolysis of thyroglobulin has been accomplished with thyroid extracts from dogs and swine (Roche *et al.*, 1950; McQuillan and Trikojus, 1953). Recently a partially purified fraction of hog thyroids has been prepared, which is rich in proteolytic activity against hemoglobin and thyroglobulin (McQuillan and Trikojus, 1953).

In the present study, the hydrolysis of rat thyroglobulin by thyroidal enzyme(s) has been investigated. Some of the conditions under which proteolytic activity occurs have been examined and the products of proteolysis have been evaluated. The method has differed from that of previous workers primarily in that it has used an extract of sliced glands and not a tissue homogenate. This extract contains principally colloidal material, and its proteolytic properties could bear relation to the *in vivo* breakdown of thyroglobulin and the release of thyroid hormone.

MATERIALS AND METHODS

Male rats[3] weighing approximately 250 grams were injected intraperitoneally with 100 microcuries of radioiodide[4] and sacrificed at 48 hours by exsanguination under ether anesthesia. The thyroids were quickly removed, trimmed, weighed on a Roller-Smith balance and frozen on dry ice. While frozen they were shaved as finely as possible with a razor blade and immersed in isotonic saline, one ml. per 30 mg. thyroid tissue, for 24 hours at

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[3] Charles River Breeding Laboratories, Boston (Sprague-Dawley strain).

[4] I^{131} was obtained from Oak Ridge and contained less than 10 μg. of carrier iodide in each 100 microcuries.

0° C. The supernatant after centrifugation (discussed hereafter as the thyroid extract) was stored in a frozen state and was utilized immediately after thawing. Frozen extract was not stored longer than three weeks.

Buffers were prepared from mixtures of the following: glycine and HCl for pH 2.35 to 3.75; acetic acid and sodium acetate for pH 3.25 to 5.97; mono- and di-sodium phosphate for pH 6.60 and 7.55; barbital and NaOH for pH 8.5. Uniform ionic strength of 0.15 was achieved by appropriate additions of NaCl. To a sample of the thyroid extract an equal volume of buffer was added and the pH of the resulting reaction solution was measured with a portable pH meter. The reaction solution was incubated at 37° C. for 24 hours (unless otherwise indicated), and then frozen until subsequent analyses were carried out. The extent of proteolysis of the thyroglobulin was assessed by salting out, dialysis, chromatography and electrophoresis.

Salting out of thyroglobulin in increasing concentrations of phosphate buffer at pH 6.5 was performed by techniques reported previously (Robbins, 1954). In the present study the concentrations of thyroglobulin used were so minute that the quantity precipitated at any small concentration change was difficult to separate. Therefore, carrier protein in the form of pooled human plasma was added to each mixture prior to salting out. Mixtures containing 0.033 ml. thyroid extract (0.066 ml. buffered extract) per ml. plasma were subjected to salting out, maintaining a final plasma dilution of 1:30. Radioactivity in the supernatant was measured with a well-type scintillation counter. Results were calculated as the per cent of radioactivity remaining in solution.

Curves for the progress of the hydrolysis with time and variation with pH were constructed, using as the estimate of hydrolysis the soluble radioactivity at 1.75 *M* phosphate buffer.

For dialysis, reaction solutions were diluted about 1:200 in isotonic saline. Measured samples (1–3 ml.) of the dilute solution were dialyzed in Visking tubing against single 17-liter volumes of isotonic saline at 4° C. for 24 hours. The loss of radioactivity from the samples was evaluated by measuring them in a scintillation counter, using as standards, identical but undialyzed volumes of the same solutions.

One-dimensional paper chromatographic analysis of the hydrolytic products was performed by techniques described earlier (Robbins and Rall, 1952), using the butanol-acetic acid system of Rosenberg (1951) and the butanol-dioxane-ammonia system of Gross *et al.* (1950). In this study, however, the chromatography strips were measured for radioactivity with a thin mica-window Geiger-Mueller tube. The radioactivity was evaluated by a continuously recording counting rate meter. Direct proportionality obtains between the area under any recorded curve and the radioactivity giving rise to it, allowing quantitation by planimetry.

Paper electrophoresis using barbital buffer at pH 8.6 was conducted as previously described (Robbins and Rall, 1952). In this study, however, the paper strips did not rest upon a glass plate, but were suspended above the plate by supports along the margins of the paper strips. The lid of the electrophoresis chamber was lined with filter paper moistened with the barbital buffer, to reduce evaporation from the strips. Human serum was added to the thyroid extract (5 volumes of serum to one of buffered extract) in order to overcome adsoption to the paper that occurred when the extract was applied alone. The strips were counted for radioactivity in the same manner as the chromatograms described above. Proteins were stained with bromphenol blue.

RESULTS

The process of extraction of the sliced thyroids in saline removed over 90% of the radioactivity. It is uncertain how much of the total proteolytic

TABLE 1. PRODUCTS OF 24-HOUR HYDROLYSIS OF VARIOUS BATCHES OF RAT THYROGLOBULIN ESTIMATED FROM BUTANOL-ACETIC ACID CHROMATOGRAMS. HYDROLYSIS WAS CONDUCTED AT 37° C., pH 3.6–3.8. AMINO ACIDS WERE IDENTIFIED BY THE USE OF STABLE CARRIERS STAINED WITH NINHYDRIN. FIGURES REPRESENT PER CENT OF TOTAL I^{131}

Remaining at the origin	Monoiodo-tyrosine	Diiodo-tyrosine	Thyroxine	Iodide and unidentified
76	8	10	4	2
69	8	12	3	8
66	9	16	8	1
66	10	16	6	2
58	11	17	8	6

activity in the colloid was extracted. However, the extent of hydrolysis was similar in various batches, as may be seen in Table 1.

The results with salting out of thyroglobulin are seen in Figure 1. The curve for unbuffered rat thyroid extract is essentially the same as that described for other mammalian thyroglobulins (Derrien *et al.*, 1948) with characteristic sharp precipitation in the region between 1.40 and 1.75 *M* phosphate buffer. Also represented is the salting out of the extract after it was buffered at pH 3.6 and incubated for 24 hours at 37° C. There is a decrease in solubility of thyroglobulin with acidification, an effect independent of hydrolysis. In samples which had been buffered but not incubated, precipitation occurred in a buffer region about 0.2 *M* less concentrated than the region for the unbuffered extract. The presence of soluble radioiodine at 1.75 *M* phosphate buffer indicates the presence of hydrolytic products comprising about 50% of the total radioactivity.

The progress of the reaction with time is shown in Figure 2. The value for soluble radioiodine at zero time was 12%. About 55% of the radioiodine was in hydrolytic products at 48 hours, after which no further hydrolysis occurred.

The variation with pH was followed in four experiments involving three batches of rat thyroglobulin (Fig. 3). Measurable hydrolysis at 24 hours was detected between pH 2.5 and 5.5. The curve was not symmetrical, dropping off sharply from the peak at pH 4.9. Another peak may be present at pH 3.5. There was no appreciable change in the pH of the reaction solutions measured before and after hydrolysis, in any portion of the pH curve. It was additionally noted that in the entire pH range over which hydrolysis occurred, the buffered extract was turbid. When the saline extract, pH 6.60 to 6.72, was incubated without buffer, no hydrolysis was observed at 24 hours.

When a sample which had been incubated at pH 3.5–5.0 was dialyzed against saline, from 35 to 45% of the radioactivity left the bag. Using unincubated samples, no activity left the bag. Dialysis prior to incubation did not affect the hydrolysis, indicating that no readily dialyzable substance is essential to the reaction.

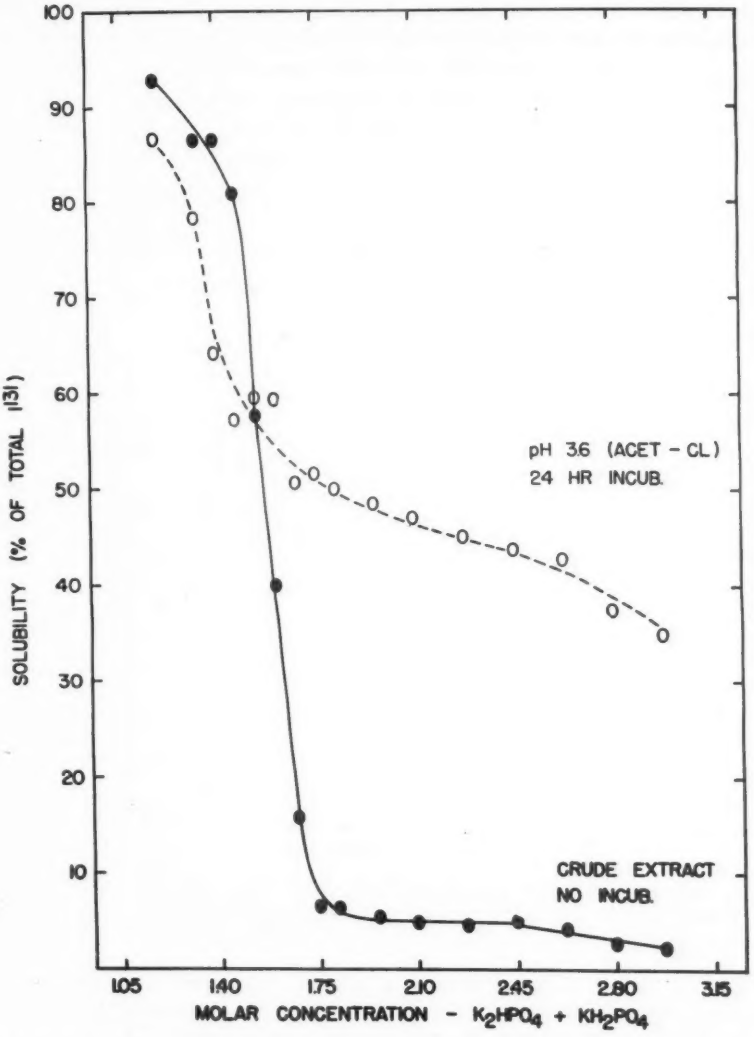

FIG. 1. Salting out of labeled rat thyroglobulin in phosphate buffer. The solid line indicates the behavior of undenatured, unhydrolysed thyroglobulin. The interrupted line represents thyroglobulin denatured by acid at pH 3.6 and incubated at 37° C. for 24 hours.

Chromatographic analysis in both acid and basic butanol systems revealed that all of the radioactivity in unincubated samples remained at the origin, which is the characteristic behavior of thyroglobulin. In the incubated samples, there were peaks of radioactivity corresponding to mono-

and di-iodo-tyrosine, and smaller amounts of iodide and thyroxine. Using the butanol-dioxane-ammonia system, which separates thyroxine and triiodothyronine, the presence of the latter was not detected in 5 batches of extract. Table 1 shows the measured products of hydrolysis from several batches of thyroglobulin. About 15% of the radioactivity was present as di-iodotyrosine, 9% as monoiodotyrosine and 6% as thyroxine. Some iodide and unidentified material was demonstrated. The results in the various batches are about the same.

The electrophoresis of unhydrolyzed thyroglobulin is seen in Figure 4.

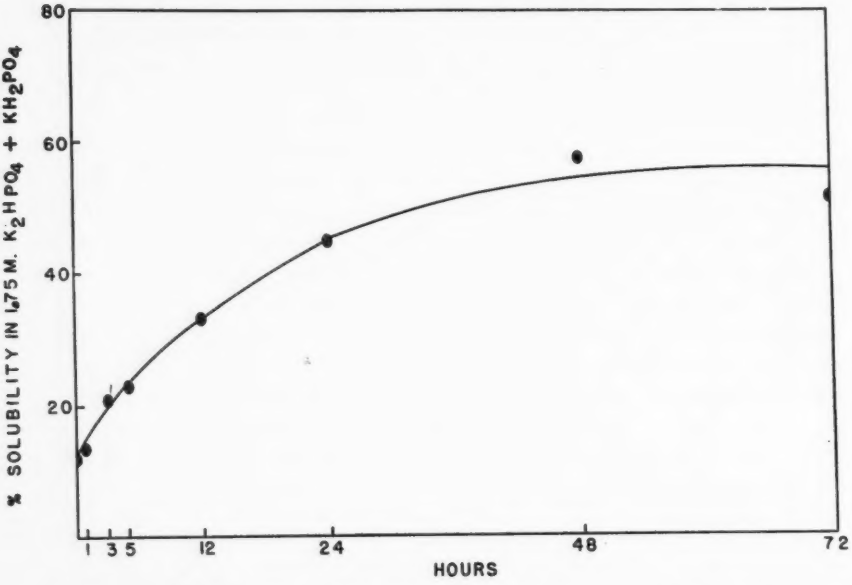

FIG. 2. The progress with time of the breakdown of rat thyroglobulin. Hydrolysis, conducted at pH 3.82 and 37° C., was measured as the per cent of radioiodine soluble in 1.75 *M* phosphate buffer

The presence of the serum proteins, in addition to allowing the labeled substance to move in a distinct band, also provided a convenient mobility reference. The upper pattern, showing a radioactive peak intermediate between alpha-1 and alpha-2 globulin, is typical of samples of thyroglobulin buffered at pH 4.6 or lower. Samples unbuffered or buffered above pH 4.6 showed an electrophoretic mobility intermediate between alpha-1 globulin and albumin, as in the lower pattern of Figure 4. It can be seen that an additional change with acidification was the presence of iodinated material at the point of application, and the streaking of a small amount of iodinated material from the major peak back to that point.

When a sample of buffered thyroglobulin was incubated for 24 hours, the major peak between alpha-1 and alpha-2 globulin, representing acidified thyroglobulin, was still seen as before incubation (Fig. 5). However, some increase in "origin" material occurred and a new peak appeared with the mobility of serum albumin.

DISCUSSION

With the method described here for the extraction of sliced thyroids in saline it was hoped to obtain the follicular contents while excluding intra-

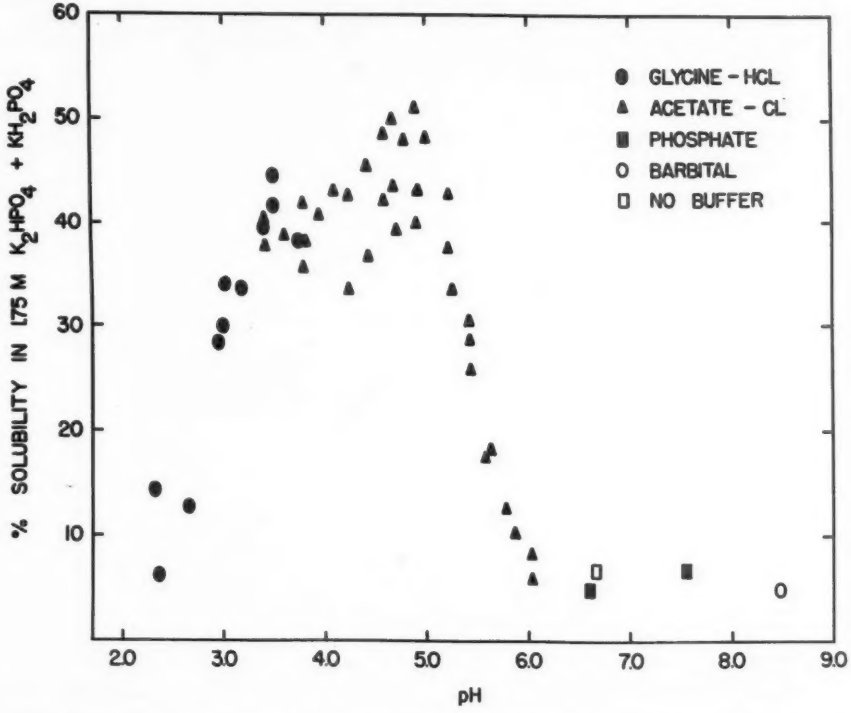

FIG. 3. The effect of various pH values upon the hydrolysis of rat thyroglobulin, conducted at 37° C., for 24 hours. Hydrolysis was measured as in Figure 2.

cellular material to a large extent. In homogenized thyroid extracts, various other enzymes have been detected with properties differing from the protease studied here. Among these are a peptidase that splits various di- and tri-peptides at pH 7.2–8.4 (Weiss, 1953); a protease that degrades gelatin with pH optimum at 6.5 (Llamas, 1951); and a nuclease which liberates phosphate from ribose nucleic acid, with a pH maximum at 5.0–5.5 (Llamas *et al.*, 1948). The presence of these other enzymes in an extract

could perhaps affect reactions like the one presently being studied. Because of the possibility that such enzymes are extracted from thyroid cells and not from the follicular colloid, it would seem desirable to avoid homogenized extracts. However, the extent of hydrolysis at 24 hours in the present method (35 to 45%) was comparable to that reported by Roche *et al.*

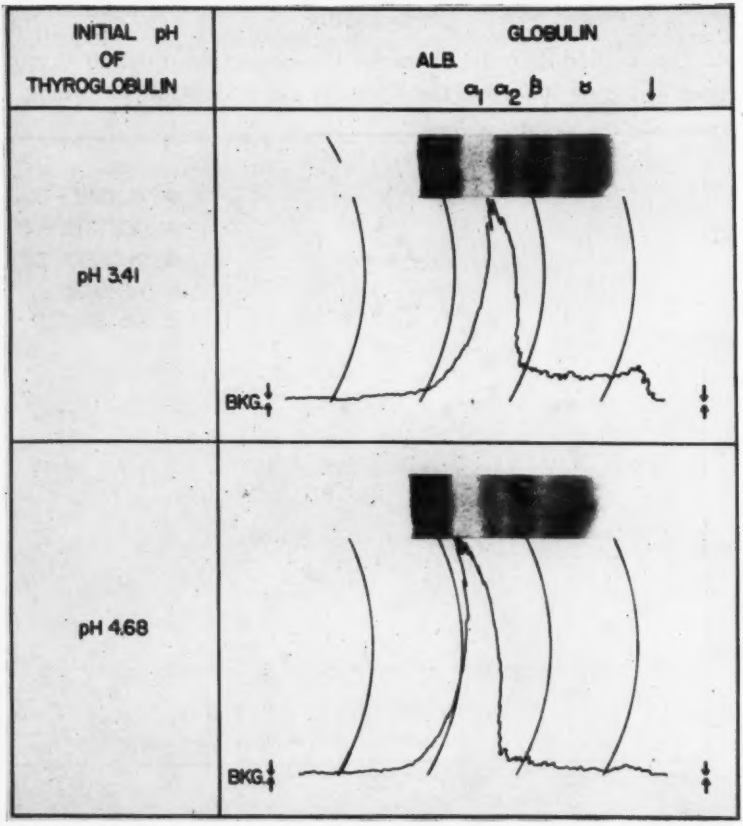

FIG. 4. Paper electrophoresis at pH 8.6 of unhydrolysed rat thyroglobulin with human serum carrier. Radioactivity recordings are mounted beneath each strip.

(1950) for homogenized extracts of thyroids of dogs and swine. It was also approximately the same as that obtained with a homogenized extract which was prepared in the present study and analysed by the same techniques as have already been described.

Denaturation of the thyroglobulin by acid was revealed in two of the analytic methods. In the salting out (Fig. 1) the denaturation of buffered samples was evidenced by a lowered solubility. In paper electrophoresis,

denatured thyroglobulin showed a decreased mobility. The lower pattern in Figure 4, with mobility between alpha-1 globulin and albumin, is that of natural thyroglobulin. The upper pattern, seen only in samples buffered at pH 4.6 or lower, is that of a denatured thyroglobulin, with mobility intermediate between alpha-1 and alpha-2 globulin. These electrophoretic

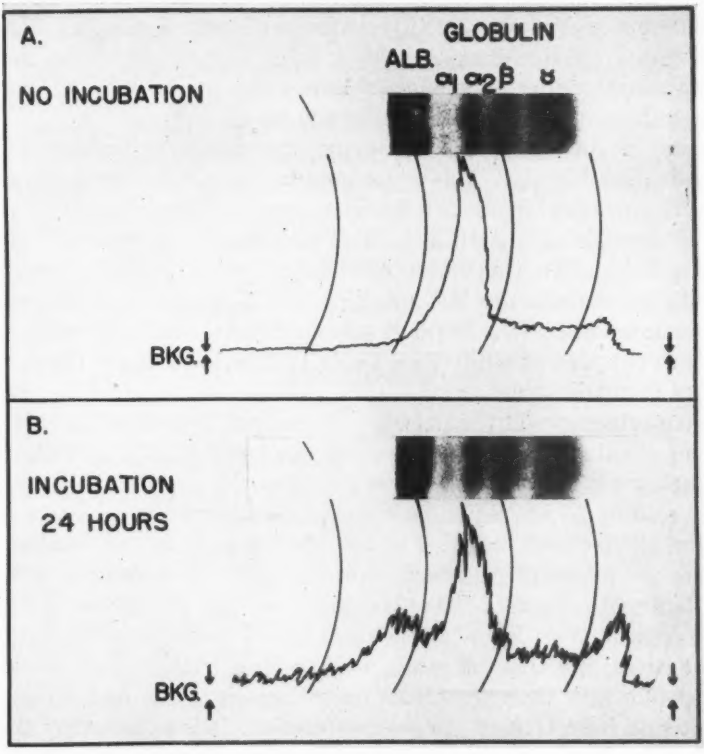

FIG. 5. Paper electrophoresis at pH 8.6 of mixtures of serum with hydrolyzed and unhydrolyzed thyroglobulin. Thyroglobulin in both samples was buffered at pH 3.82. Serum was added just prior to electrophoresis.

findings are in agreement with those of Heidelberger and Pedersen (1935), using moving boundary electrophoresis.

The curve for the salting out of unbuffered thyroglobulin (Fig. 1) demonstrates the suitability of this method for the characterization of rat thyroglobulin by radioactive means. Assuming that the thyroglobulin concentration in wet thyroid tissue is about 10%, the saline extract contained approximately 3 mg. of thyroglobulin per ml. With the salting out method, in which a 1:1000 dilution of this extract was used, the final concentration

of thyroglobulin was a few μg. per ml. In the face of this minute concentration, separation of the precipitated thyroglobulin was still possible, because carrier protein was added. As noted previously (Robbins, 1954), success with this technique is favored by the exceedingly low solubility of thyroglobulin at the higher concentrations of neutral salts, even when present itself in the low concentrations indicated. The presence of serum does alter the pattern of precipitation of thyroglobulin in neutral salts. This has been demonstrated by Roche *et al.* (1951), using hog thyroglobulin and ammonium sulfate buffer. The effect shown was a minor one, however, and did not influence the main features of the curve.

The use of the salting out technique to estimate hydrolysis (whereby radioiodine soluble at 1.75 *M* phosphate buffer is considered to reside in hydrolytic products) involves a few inaccuracies. First, the measured solubility of thyroglobulin itself at 1.75 *M* phosphate is about 10% (Fig. 1). Secondly, it is not known to what extent the hydrolytic products may combine with plasma proteins in the salting out. Thyroxine in serum is about 30% precipitated at 1.75 *M* phosphate (Robbins, 1954), but it is a small product in the present study (See Table 1). The behavior of the iodinated tyrosines is not known.

The true character of the pH curve is unclear, primarily because of the variation of values at any one pH from one batch of thyroglobulin to another. Below pH 3.6 the buffering capacity of the acetate system is small, but the results in this region compared favorably with values at similar pH using glycine-HCl buffer. The fact that the pH of the reaction solutions was not apparently altered in the course of the hydrolysis, even in a poorly buffered portion of the curve, indicates that the buffering capacity was not critical. With regard to the turbidity of the buffered extract, noted over the entire effective pH range, it is possible that varying amounts of thyroglobulin may thus have been out of solution, and hydrolyzing at a different rate from that of the soluble fraction. It is noteworthy that the highest values occurred at pH 4.9, in a region where thyroglobulin is not denatured by acid. Electrophoretic evidence has been obtained at this pH, however, to indicate that denaturation of the substrate is a primary step in the enzymatic process. These problems are being investigated further.

The significance of the electrophoretic changes observed after the hydrolysis of an extract is unclear, even in the light of the products identified by chromatography. It is likely that the new peak of radioactivity with the mobility of serum albumin is due at least in part to the production of di-iodotyrosine, which is the major product. The actual binding behavior of the iodotyrosines with serum proteins is not known, however. Thyroxine is known to bind with both albumin and the alpha-globulin fraction, but it is quantitatively a small product here. Further work is contemplated to clarify these findings.

SUMMARY

A mild saline extract containing thyroglobulin and a proteolytic enzyme was prepared from the thyroid glands of rats injected with radioiodide. The extent of hydrolysis of thyroglobulin after incubation of this extract has been assessed by salting out, dialysis, chromatography and electrophoresis.

As measured by salting out techniques, the hydrolysis of thyroglobulin occurred between pH 2.5 and 5.5, with peak activity at pH 4.9. Fifty-five per cent of the thyroglobulin was hydrolyzed at 48 hours, after which no further hydrolysis was observed. By paper chromatography, thyroxine, mono- and di-iodotyrosine and iodide have been identified as products. Triiodothyronine was not detected.

The salting out curve for rat thyroglobulin in phosphate buffer is similar to that described for other species.

Acknowledgment

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REFERENCES

De Robertis, E.: *Anat. Rec.* **80**: 219. 1941.
Derrien, Y., R. Michel and J. Roche: *Biochim. et biophys. acta* **2**: 454. 1948.
Dziemian, A. J.: *J. Cell. & Comp. Physiol.* **21**: 339. 1943.
Gross, J., C. P. Leblond, A. E. Franklin and J. H. Quastel: *Science* **111**: 605. 1950.
Heidelberger, M., and K. O. Pedersen: *J. Gen. Physiol.* **19**: 95. 1935.
Kamner, M. E., A. Peranio and M. Bruger: *Endocrinology* **46**: 353. 1950.
Llamas, R.: *An. Inst. Biol. (Univ. Nacl., Mex.)* **21**: 269. 1950; (Chem. Abstr. **45**: 6229 f. 1951.)
Llamas, R., J. Roca and R. Ondarza: *An. Inst. Biol. (Univ. Nacl., Mex.)* **18**: 15. 1947; (*Chem. Abstr.* **42**: 625a. 1948.)
McQuillan, M. T. and V. M. Trikojus: *Australian J. Biol. Sc.* **6**: 617. 1953.
Robbins, J.: *J. Biol. Chem.* **208**: 377. 1954.
Robbins, J. and J. E. Rall: *Proc. Soc. Exp. Biol. & Med.* **81**: 530. 1952.
Roche, J., G. Deltour, R. Michel and S. Lissitzky: *Compt. rend. Soc. biol.* **144**: 1647. 1950.
Roche, J., R. Michel, O. Michel, G. Deltour and S. Lissitzky: *Biochim. et biophys. acta* **6**: 572. 1951.
Rosenberg, I. N.: *J. Clin. Investigation* **30**: 1. 1951.
Weiss, B.: *J. Biol. Chem.* **205**: 193. 1953.

NOTES AND COMMENTS

EVIDENCE FOR PITUITARY CONTROL OF THE LIPID CONTENT OF THE ZONA GLOMERULOSA OF THE RAT ADRENAL CORTEX

The investigations of Greep and Deane (1949) have indicated that the zona glomerulosa of the rat adrenal cortex elaborates DOC-like steroids. These compounds are held to be involved in mineral metabolism. Experimental animals in the hypophysectomized state do not require salt as a dietary supplement. This fact, coupled with histological evidence for the integrity of this zone, despite atrophy of the inner zones, for prolonged periods following hypophysectomy, suggests independence of pituitary control. The ACTH preparations employed by Bergner and Deane (1948) and Ingle *et al.* (1947) appeared to have little or no effect on electrolyte metabolism in the rat. The inference may be drawn that the pituitary components present in these preparations were without effect on the zona glomerulosa.

Conversely, clinical and experimental studies in man have shown that various ACTH preparations will induce profound alterations in mineral metabolism, (Sprague, R. G. and Power, M. H., 1950). If the species are comparable, and the zona glomerulosa of rat adrenal is, in fact, involved in the production of the mineralocorticoids, it might be expected that preparations would be encountered which would produce evidence suggesting activity in this zone. Over a period of several years we have found this to be the case. Adrenal glands obtained from hypophysectomized rats, treated with various commercial ACTH preparations, have exhibited alterations in the cytology and pattern of lipid distribution in the zona glomerulosa. Further, we have observed depletion of sudanophilic material in this zone in the adrenals of intact rats subjected to certain stimuli, i.e., temperature fluctuation and nitrogen mustard. (H. R. and A. G.) Similar observations have been made in the zona glomerulosa of the mouse by Jones (1949) using other stimuli.

MATERIALS AND METHODS

The results herein described were obtained over a period of several years. The animals used were of both sexes of the Long-Evans or Sprague Dawley strains. Body weights ranged from 40 to 300 gm. Hypophysectomy was performed by the parapharyngeal approach. All animals were examined upon sacrifice for completeness of hypophysectomy.

Commercial ACTH preparations were used, including highly potent materials (e.g., Armour's and Wilson's adrenocorticotrophin purchased from local outlets), made up in diluents including saline, phenol, lactate, dilute acetic acid, gelatin, etc. In addition, corticotrophic extracts, prepared by ourselves and others, were studied. In the majority of cases injections were given subcutaneously, once daily. Animals were sacrificed at various time intervals after hypophysectomy, i.e., at daily and at weekly intervals. The experiments of longer duration extended for 21 days.

Adrenal glands preserved for histological analysis were fixed in 10% neutral formalin (Lillie), embedded in gelatin and cut at various thicknesses (6 to 10μ). Frozen sections were stained with sudan Orange and hematoxylin, or with sudan black B and carmalum.

RESULTS

Within 24 to 48 hours after hypophysectomy a definite increase in the width of the zona glomerulosa was observed. There was marked proliferation of cells between the capsule and the band of lipid-rich cells adjacent to it. The former had little or no cytoplasmic lipid. The latter retained their lipid, although there was evident change in its physical appearance

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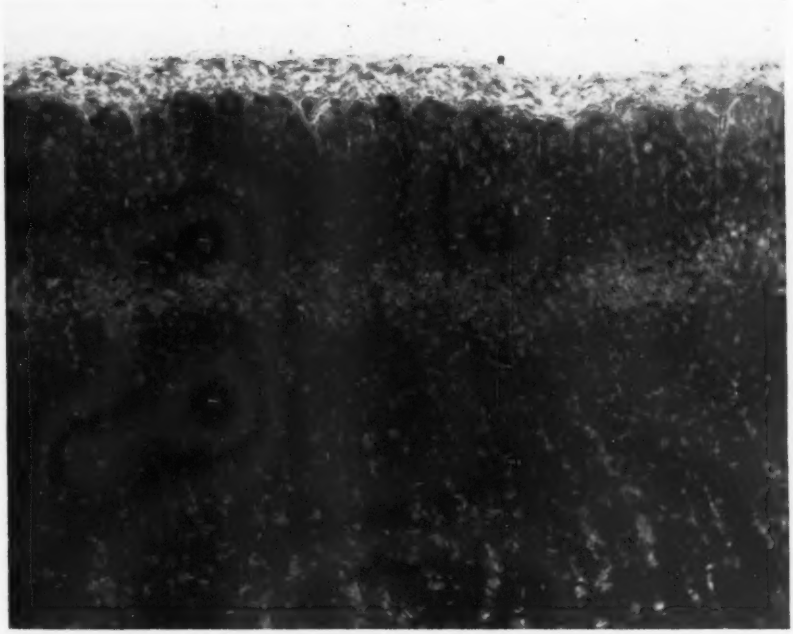

FIG. 1. Adrenal cortex of rat given 3 subcutaneous injections of 0.5 ml. physiological saline, daily, beginning 24 hours post hypophysectomy. Note zona glomerulosa is full of lipid with a lipid-free zone between glomerulosal and fasciculate zones. Sudan Orange and Hematoxylin, 10 μ., $\times$270.

Untreated animals or animals given nonspecific treatment, such as saline, gelatin, etc., yielded adrenals with glomerulosal zones broadened following hypophysectomy. In this state the lipid content of the zone remained inert, while the lipid-free zone between it and the zona fasciculata increased in width.

Following treatment with some, but not all, ACTH preparations (in doses up to one I.U. per 100 gm. of body weight) adrenals were obtained showing lipid-depleted zonae glomerulosa, in contrast to the controls. The process of lipid depletion in this zone was observed to be progressive. For example, when the animals were given a single injection of ACTH lipid depletion was detected within a few hours. Depletion, however, was not complete until successive injections had been made. In experiments where the animals were sacrificed following 2 or 3 days' injections the zona glomerulosa was in many cases entirely depleted of lipid. Such depletion was observed in the adrenals of animals receiving high potency ACTH preparations in gelatin, although its appearance was delayed by a day or more in contrast to the animals receiving hormonal material without gelatin. No evidence of lipid reaccumulation during treatment was obtained. Intact experimental rats, about 125 gm. in body weight, receiving nitrogen mustard in a single injection of 0.5 to 1.5 mg., also yielded glands, 72 hours after injection, in which the zona glomerulosa was entirely depleted of sudanophilic material, although, in these experiments, complete or almost complete depletion was noted as well in the zona fasciculata and zona reticu-

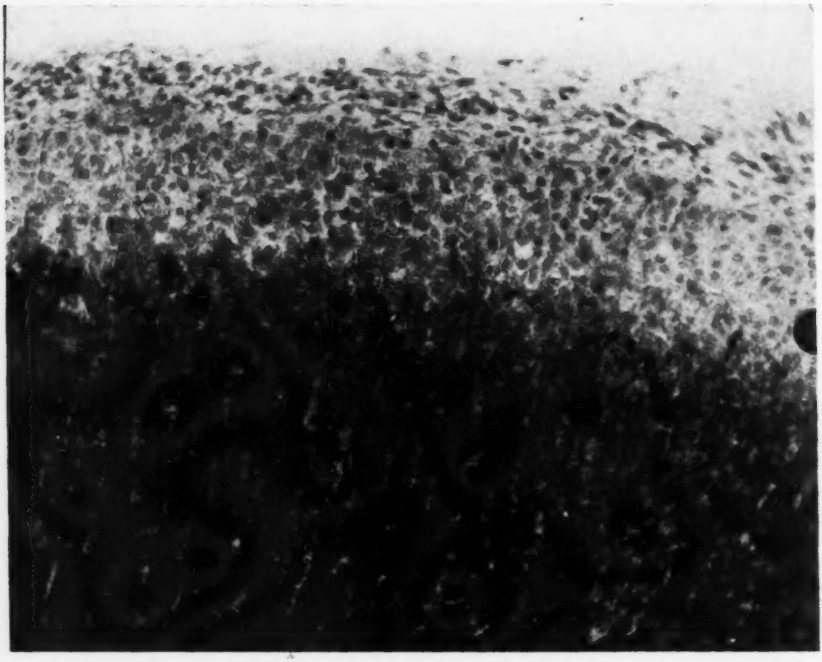

FIG. 2. Adrenal cortex of rat given 3 subcutaneous injections of 2/3 I.U. ACTH (in gelatin) daily, beginning 24 hours post hypophysectomy. Note complete disappearance of lipid from zona glomerulosa.

laris. This same treatment when carried out in hypophysectomized rats produced no depletion in the zona glomerulosa.

Intact animals, sacrificed following maintenance in a room subject to extremes of summer day and night temperatures, and receiving no other treatment, were observed to possess adrenals depleted of glomerulosal lipid.

DISCUSSION

No explanation is offered for the increase in the width of the zona glomerulosa immediately following hypophysectomy in the rat. We have speculated that during the course of the surgical process a pituitary substance is released which stimulates for a time the reproduction of secretory units in this zone. The continued existence of the zone during the hypophysectomized state, without atrophy, would suggest that the maintenance of structural relationships within the zone is not dependent upon material of pituitary origin. On the other hand, the secretory activity of the zona glomerulosa, as indicated by the change in its content of sudanophilic material, appears to be subject, at least in part, to pituitary control. That it has been our experience to observe these changes following the administration of some ACTH preparations, but not others, suggests a difference in their composition. However, their pituitary origin reinforces the concept of hypophyseal influence on the zona glomerulosa of the rat adrenal cortex. This

would not imply that factors other than those arising in the pituitary may not produce modifications in activity in this zone.

The evidence obtained with intact rats treated with nitrogen mustard, when compared with that obtained from hypophysectomized rats similarly treated, suggests that certain types of stimuli result in the secretion by the pituitary of components capable of discharging the lipid content of the zona glomerulosa. This possibility is likewise indicated in the zona glomerulosal lipid distribution in animals maintained under conditions of fluctuating temperature.

Whether or not the observations made in these experiments involving ACTH are related to clinical indications of altered electrolyte metabolism in man remains to be determined. The evidence provided by Greep and Deane, (1949), wherein the elaboration of DOC-like steroids appears to be a function of the zona glomerulosa, warrants the proposal that such a study would yield information of interest.

SUMMARY

Complete depletion of sudanophilic material in the zona glomerulosa of the adrenal cortex has been observed in hypophysectomized rats receiving various ACTH preparations. This phenomenon is not necessarily related to lipid depletion in the other zones of the cortex. A similar loss of staining material has been demonstrated in the zona glomerulosa of intact rats treated with nitrogen mustard, although, in this case, extensive depletion in the zona fasciculata and zona reticularis has frequently accompanied this finding. Adrenals obtained from hypophysectomized rats treated with nitrogen mustard have not exhibited depletion of lipid in the zona glomerulosa. Rats maintained under conditions of fluctuating day and night temperatures have yielded adrenals showing evidence of lipid mobility in the zona glomerulosa.

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REFERENCES

Bergner, G. E. and H. W. Deane: *Endocrinology* **43**: 240. 1948.
Greep, R. O. and H. W. Deane: *Ann. New York Acad. Sc.* **50**: 596. 1949.
Ingle, D. J., M. C. Prestrud, C. H. Li and H. M. Evans: *Endocrinology* **41**: 170. 1947.
Jones, I. C.: *Endocrinology* **45**: 5, 4. 1949.
Sprague, R. G. and M. H. Power, Pituitary-Adrenal Function, AAAS, Washington, D. C., 1950.

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THE 1955 ANNUAL MEETING

The Thirty-seventh Annual Meeting of The Endocrine Society will be held in the Chalfonte-Haddon Hall Hotel, Atlantic City, New Jersey, on Thursday, Friday and Saturday, June 2, 3 and 4, 1955.

Dr. Matthew Molitch, 705 Pacific Avenue, Atlantic City, New Jersey, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Chalfonte-Haddon Hall Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 3, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately, as the hotels expect to be filled to capacity. Correspond directly with Chalfonte-Haddon Hall, advising time of arrival and departure. Make your reservations now and avoid disappointment.

Those wishing to present papers, (time limited to ten minutes) should send four copies of the title and abstract to the Vice-President, Dr. E. B. Astwood, 30 Bennet Street, Boston 11, Massachusetts, not later than *February 1, 1955*. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. Neither footnotes nor acknowledgments to sponsors can be published. References, if used, should be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading should be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each nonmember-author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of nonmembers who are introduced, *i.e.*, who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, *e.g.*, M.D., of each author should be given after his name.
   Line 3. Institute of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, as there will be no opportunity for proof reading by authors.
5. Abstracts not conforming to the foregoing regulations will be returned to the authors.